COMPOSITIONS AND METHODS FOR TREATING HEART DISEASE

FIELD OF THE INVENTION

The invention relates to compositions and methods for treating heart disease by inhibition of PI3Kgamma.

BACKGROUND OF THE INVENTION

Cardiovascular diseases are predicted to be the most common cause of death worldwide by 2020 (Yusuf et al, 2001). Cardiovascular disease is frequently associated with elevated wall stress as a result of pressure overload such as in hypertension or volume overload seen in valvular disorders. Increased wall stress in the heart triggers a hypertrophic response (Hunter and Chien, 1999). What is initially a compensatory response, pathological hypertrophy eventually leads to decompensation resulting in left ventricle dilation, myocyte loss, increased interstitial fibrosis and heart failure. While increased wall stress can lead to pathological cardiac hypertrophy, increased demand for cardiac output can also cause a physiological increase in cell size, as commonly seen in endurance athletes (Colan, 1997).

In flies and mammals, hypertrophic responses can be initiated via phosphoinositide 3-kinase (PI3K) signaling pathways (Leevers et al, 1996; Shioi et al, 2000). PI3Ks constitute a family of evolutionarily conserved lipid kinases that regulate a vast array of fundamental cellular responses, including proliferation, adhesion, cell size, and protection from apoptosis (Toker and Cantley, 1997; Stephens et al, 1993). These responses result from the activation of membrane trafficking proteins and enzymes such as the phosphoinositide-dependent kinases (PDKs), PKB/Akt, or S6 kinases by the key second messenger PIP3 (Franke et al, 1997; Alessi et al, 1998; Downward, 1998). Four different type I PI3Ks have been described three of which (PI3Kα, β, δ) are activated by receptor tyrosine kinase pathways. PI3Kγ is activated by the $\beta\gamma$ subunit of G-proteins and acts downstream of G-protein-coupled receptors (GPCRs) (Toker and Cantley, 1997). Recent genetic evidence in haematopoietic cells indicates

that PI3Kγ is the sole PI3K that couples to GPCRs (Sasaki et al, 2000; Hirsch et al, 2000; Li et al, 2000).

In isolated cardiomyocytes, PI3K signaling has been implicated as a component of cardiac hypertrophy and protection of myocytes from apoptosis mediated by numerous exogenous agonists and stresses (Rabkin et al, 1997; Schluter et al, 1998; Baliga et al, 1999; Kodama et al, 2000). In transgenic mice, overexpression of constitutively active p110α results in increased heart size, whereas cardiac overexpression of dominant negative p110α in mice resulted in smaller hearts without affecting heart functions (Shioi et al, 2000). Furthermore, PI3Kγ activity is increased upon aortic constriction in mice (Naga Prasad et al, 2000), suggesting that both tyrosine-based and GPCR-linked PI3Ks might play a role in the cardiac hypertrophy response.

The tumor suppressor PTEN is a lipid phosphatase that dephosphorylates the D3 position of PIP3 (Maehama and Dixon, 1998). Thus, PTEN lowers the levels of the PI3K product PIP3 within the cells and antagonizes PI3K mediated cellular signaling. It has been recently shown that PTEN can regulate neuronal stem cell proliferation and the cell size of neurons in brain-specifc PTEN mouse mutants similar to patients with Lhermitte-Duclos disease (Backman et al, 2001). Moreover, expression of dominant negative PTEN gene in rat cardiomyocytes in tissue culture results in hypertrophy (Schwartzbauer and Robbins, 2001). Whether PI3Ks and PTEN can indeed control cardiac hypertrophy and heart functions *in vivo* is not known.

Summary of the Invention

The inventors have identified PTEN as a master regulator for heart size and heart function and PI3kgamma as a critical regulator of heart muscle function (pumping function) *in vivo* in the whole mouse (mutant mouse model) and *in vitro* in single cells. These are completely new and unexpected findings and identify novel pathways to protect from heart failure. Inhibition of PI3Kgamma and the novel PI3Kgamma signaling pathway allows maintenance of heart function even in failing hearts. Inhibition of PI3Kγ and/or activation of PTEN blocks cardiac hypertrophy. The invention describes a novel and previously unrecognized function for the

PI3Kgamma/PTEN pathways *in vivo*. The functionality was shown *in vivo* in the whole organism and at the single cell level. Gene targeting in mice using multiple mutants and in a heart failure model was used to establish the *in vivo* role of PI3Kgamma and PTEN. The invention studied *PTEN*-heart muscle specific mutant mice, $p110\gamma^{-/-}$, dominant-negative *p110α* transgenic mice, and double-mutant mice. Two independent PI3K signaling pathways exist in cardiomyocytes that can be genetically uncoupled. While the tyrosine kinase-receptor p110α-PTEN pathway is a critical regulator of cardiac cell size, the GPCR-linked PI3Kγ-PTEN signaling pathway modulates heart muscle contractility.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the invention will be described in relation to the drawings in which:

Figure 1. Cardiac Hypertrophy in PTEN-mutant hearts

A, Western blot analysis of proteins from different tissues of *mckCRE-PTEN*$^{\Delta/+}$ and *mckCRE-PTEN*$^{\Delta/\Delta}$ mice.

B, Western blot analysis for p110α, p110β, p110γ, and p85 expression levels in total heart lysates from 2 representative *mckCRE-PTEN*$^{\Delta/+}$ and two *mckCRE-PTEN*$^{\Delta/\Delta}$ mice.

C, Representative heart sizes of 10 week old *mckCRE-PTEN*$^{\Delta/+}$ and *mckCRE-PTEN*$^{\Delta/\Delta}$ littermates.

D, Heart sections from 10 week (upper and middle panels) and 1 year (lower panels) old *mckCRE-PTEN*$^{\Delta/+}$ and *mckCRE-PTEN*$^{\Delta/\Delta}$ mice. The middle and lower panels show higher magnifications to detect interstitial fibrosis (trichrome). LV = left ventricle; RV = right ventricle.

Figure 2. Cardiac Hypertrophy and PI3K activation

A, Quantitation of heart/body weight ratios from 10 week old *mckCRE-PTEN*$^{\Delta/+}$ (n=8) and *mckCRE-PTEN*$^{\Delta/\Delta}$ (n=9) and 12 month old *mckCRE-PTEN*$^{\Delta/+}$ (n=6) and *mckCRE-PTEN*$^{\Delta/\Delta}$ (n=6) littermate mice.

B, Increased cell sizes of cardiomyocytes isolated from 10 week old *mckCRE-PTEN$^{\Delta/\Delta}$* mice. Representative images of isolated cardiomyocytes and quantitation of cell sizes are shown. 100 individual cells from three different mice were analyzed per group. ** $p < 0.01$ between genetic groups.

C,D Northern blot analysis and quantitation (mean values +/- SEM) of cardiac hypertrophy markers. GAPDH expression levels were used as a loading control. Results from hearts isolated from 3 individual 10 week old *mckCRE-PTEN$^{\Delta/+}$* and 3 different, age-matched *mckCRE-PTEN$^{\Delta/\Delta}$* littermate mice are shown.

E,F Increased AKT/PKB, GSK3β, and $p70^{S6K}$ phosphorylation in total heart extracts from 10 week old *mckCRE-PTEN$^{\Delta/\Delta}$* mice. Western blot of the expression of phospho-AKT/PKB, phospho-GSK3β, phospho-$p70^{S6K}$, phospho-ERK1/2, and their respective loading controls. Densitometric quantitation of phospho-AKT/PKB and phospho-GSK3β levels relative to total cellular AKT/PKB and GSK3β. Mean values +/- SEM are representative of 3 independent experiments. ** $p < 0.01$ between genetic groups.

Figure 3. Modulation of cardiac contractility in PTEN and p110γ mutant hearts

A, Left ventricular mass (LVM; mean values +/- SEM) in hearts from 10 week old *mckCRE-PTEN$^{\Delta/\Delta}$* (n=8), *wild-type* (n=7), *p110γ$^{-/-}$* (n=7), and *mckCRE-PTEN$^{\Delta/\Delta}$ p110γ$^{-/-}$* (n=8) mice. ** $p < 0.01$ between genetic groups.

B, Percent fractional shorting (%FS) and velocity of circumferential fibre shortening (Vcfs) in 10 week old *mckCRE-PTEN$^{\Delta/\Delta}$*, *wild-type*, *p110γ$^{-/-}$*, and *mckCRE-PTEN$^{\Delta/\Delta}$ p110γ$^{-/-}$* double mutant mice. Mean values +/- SEM were determined by echocardiography. ** $p < 0.01$ between groups.

C, M-mode echocardiographic images of contracting hearts in 10 week old *mckCRE-PTEN$^{\Delta/\Delta}$*, *wild-type*, *p110γ$^{-/-}$*, and *mckCRE-PTEN$^{\Delta/\Delta}$ p110γ$^{-/-}$* mice. Note that contracting hearts in *mckCRE-PTEN$^{\Delta/\Delta}$ p110γ$^{-/-}$* double mutants are similar to that of *p110γ$^{-/-}$* mice.

D. Western blot analysis for p110γ expression in control and p110γ deficient isolated cardiomyoctes. Note the smaller non-specific band.

E, Western blotting for phosphorylated-AKT/PKB, total AKT/PKB protein, p85, p110α, and p110β protein expression in total heart extracts.

F, Representative heart sizes and heart/body weight ratios of 10 week old *p110γ*$^{+/-}$ and *p110γ*$^{-/-}$ littermates. Similar data, i.e, no change in heart size, were obtained at 6 and 12 months of age.

G, Western blot analysis of AKT/PKB phosphorylation and total AKT/PKB protein levels in hearts from individual *p110γ*$^{-/-}$, *mckCRE-PTEN*$^{Δ/Δ}$ and *mckCRE-PTEN*$^{Δ/Δ}$ *p110γ*$^{-/-}$ double mutant mice.

Figure 4. Heart sizes and functions in *mckCRE-PTEN*$^{Δ/Δ}$ *DN-p110α* mice

A, Representative heart sizes of 10 week old *wild-type, DN-p110α,* and *mckCRE-PTEN*$^{Δ/Δ}$ *DN-p110α* littermates.

B, Left ventricular mass (LVM; mean values +/- SEM) in hearts from 10 week old *mckCRE-PTEN*$^{Δ/Δ}$ (n=8), *wild-type* (n=6), *DN-p110α* (n=4), and *mckCRE-PTEN*$^{Δ/Δ}$ *DN-p110α* double transgenic (n=4). ** $p < 0.01$ between genetic groups.

C, Western blot analysis of AKT/PKB phosphorylation and total AKT/PKB protein levels in hearts from *wild type* (lane 1), *DN-p110α* single transgenic (lane 2), and *mckCRE-PTEN*$^{Δ/Δ}$ *DN-p110α* double transgenic (lane 3) mice.

D, M-mode echocardiographic images of contracting hearts in 10 weeks old *wild type*, *mckCRE-PTEN*$^{Δ/Δ}$, and *mckCRE-PTEN*$^{Δ/Δ}$ *DN-p110α* double transgenic mice.

E, Percent fractional shorting (%FS) and velocity of circumferential fibre shortening (Vcfs) in 10 weeks old *mckCRE-PTEN*$^{Δ/Δ}$ (n=8), *wild-type* (n=6), *DN-p110α* (n=4), and *mckCRE-PTEN*$^{Δ/Δ}$ *DN-p110α* double transgenic (n=4) mice. Values (mean +/- SEM) were determined by echocardiography. ** $p < 0.01$ between groups.

Figure 5. Single cell contractility and cAMP production

A, B, Basal contractility of single cardiomyocytes isolated from *mckCRE-PTEN$^{\Delta/\Delta}$*, *wild-type*, *p110$\gamma^{-/-}$*,and *mckCRE-PTEN$^{\Delta/\Delta}$ p110$\gamma^{-/-}$* double mutant mice. In **A**, representative contractions from single cells are shown. In **B,** mean percentage shortening (+/- SEM) of at least 15 different single cells from 3 different hearts per genotype are shown in the presence and absence of thePI3K inhibitor LY294002 [30μM]. ** $p < 0.01$ between groups.

C, Basal cAMP levels (mean +/- SEM) in purified cardiomyocytes from *mckCRE-PTEN$^{\Delta/\Delta}$*, *wild-type*, and *p110$\gamma^{-/-}$* mice (n = 4 for each genotype). Samples were either left untreated (-) or treated with wortmannin (wort, +). * $p < 0.05$ between groups.

D, Activation of adenylate cyclase via forskolin [25μM] increases the levels of cAMP production (mean +/- SEM) in *mckCRE-PTEN$^{\Delta/\Delta}$*, *wild-type,* and *p110$\gamma^{-/-}$* cardiomyocytes to a similar extent.

E, Inhibition of cAMP dependent functions via Rp-cAMPS [25μM] reduces cell shorting (contractility) in single *wild-type* and *p110$\gamma^{-/-}$* cardiomyocytes. Mean values (+/- SEM) of percent shortening in the presence of Rp-cAMPS (left panel) and percent decrease in contractility using Rp-cAMPS are shown. ** $p < 0.01$ between groups.

Figure 6. PI3Kγ and β-adrenergic signaling

A, GRK kinase activities from fractionated heart lysates from individual *mckCRE-PTEN$^{\Delta/+}$* and *mckCRE-PTEN$^{\Delta/\Delta}$* mice were determined using in vitro kinase assays and rhodopsin as substrate. Since GRKs are recruited to membranes, data from cytosolic and membrane fractions are shown. C = control using recombinant GRK2. The lower panel shows a Western blot of total GRK2/3 expression levels in the hearts.

B, β-AR densities were determined using ICYP as a ligand as described in Experimental Procedures (n = 7 for *wild type*; n = 5 for *mckCRE-PTEN$^{\Delta/\Delta}$* and n = 5 for *p110$\gamma^{-/-}$*).

C, Induction of cAMP production in response to the selective β2-adrenergic receptor agonist zinterol. Note that zinterol has no detectable effect on β2-AR induced cAMP levels in *wild type* cardiomyocytes (n = 5) but significantly increases cAMP levels in $p110\gamma^{-/-}$ cardiomyocytes (n = 5). * $p < 0.05$ between groups.

D, Induction of cAMP production in response to β1-adrenergic receptor stimulation. Both *wild type* (n = 4) and $p110\gamma^{-/-}$ cardiomyocytes (n = 4) respond equally to isoproterenol. β2-AR were blocked with pretreatment of ICI 118,551.

E, Western blot analysis of phosphorylated phospholamban (P-PLB) and total phospholamban (PLB) in *wild-type* and $p110\gamma^{-/-}$ cardiomyocytes in response to different concentrations of the selective β2-AR agonist zinterol. C = control. Note the increased phosphorylation of PLB in unstimulated $p110\gamma^{-/-}$ cardiomyocytes that mirrors increased basal cAMP levels in these cells. One representative experiment is shown.

Figure 7. a) Homo sapiens phosphoinositide-3-kinase gamma nucleic acid sequence [SEQ ID NO: 1]; Accession no. AF327656; base count: 950 a 861 c 849 g 750 t b) Homo sapiens phosphoinositide-3-kinase gamma amino acid sequence[SEQ ID NO: 2].

Figure 8. a) Homo sapiens PTEN nucleic acid sequence[SEQ ID NO: 3]; Accession no. NM_000314; base count: 853 a 742 c 788 g 777 t; b) Homo sapiens PTEN amino acid sequence [SEQ ID NO: 4].

Figure 9. Signaling and cardiac hypertrophy responses in $p110\gamma^{+/-}$ and $p110\gamma^{-/-}$ mice. A) Heart sections from $p110\gamma^{+/-}$ and $p110\gamma^{-/-}$ mice treated with a vehicle control or isoproterenol for 7 days. B) Quantitation of heart/body weight and heart weight/tibial length ratios in vehicle and isoproterenol treated mice; n=9 per group. *P<0.05; **P<0.01 compared with vehicle. C) Northern blot analysis and quantitation of cardiac hypertrophy markers in $p110\gamma^{+/-}$ and $p110\gamma^{-/-}$ mice treated with vehicle (1) or isoproterenol (2) for 7 days. GAPDH expression levels were used as a loading control. It should be noted that the baseline levels of the indicated markers were comparable between $p110\gamma^{+/-}$ and $p110\gamma^{-/-}$ hearts. D) ERK1/2 and p38 phosphorylation in isolated $p110\gamma^{+/-}$ and $p110\gamma^{-/-}$ neonatal cardiomyocytes. Cells were

(1) left untreated, or stimulated with (2) isoproterenol (10μM) and (3) basic fibroblast growth factor (bFGF) (250 ng/ml) for 10 mins. Western blot data are shown. E) Impaired Akt/PKB activation in total heart extracts from $p110\gamma^{+/-}$ and $p110\gamma^{-/-}$ mice treated with vehicle control or isoproterenol for 7 days; n=5 per group. *P< 0.01 between all groups. Values are mean±SEM.

Figure 10. Loss of p110γ protects from interstitial fibrosis and hypertrophy. A) Heart sections from $p110\gamma^{+/+}$ and $p110\gamma^{-/-}$ mice treated with a vehicle control or isoproterenol for 7 days. Trichrome and picro sirius red (PSR) staining to detect remodeling and interstitial fibrosis are shown. B) Morphometric quantitation of fibrosis in vehicle and isoproterenol treated mice. n=5 per group. *P<0.01 compared with vehicle. C) Quantitation of apoptosis in vehicle and isoproterenol treated mice. n=3 per group with 3 sections from each heart. *P<0.05 compared with vehicle. D) Quantitation of cardiomyocyte size based on cross-sectional area in vehicle and isoproterenol treated mice. n=3 per group with 3 sections from each heart. *P<0.05; **P<0.01 compared with vehicle. Values are mean±SEM.

Figure 11. Ventricular β-adrenergic receptor densities. A) Immunoblots on purified sarcolemmal preparations (PM). PMs were enriched in the specific markers such as Na-K ATPase, as well as caveolin-1 and caveolin-3 (not shown). Immunoblot analysis was with anti-type V/VI adenylyl cyclase (*upper panel*, 20 mg protein loaded), anti-β_1-AR (*middle panel*, 20 μg protein loaded) or anti-β_2-AR (*bottom panel*, 100 mg protein loaded) antibodies or following antibody preblocking with antigen (+Antigen). Protein recovery was similar for $p110\gamma^{+/-}$ and $p110\gamma^{-/-}$ ventricles without or with isoproterenol treatment. B) Quantifications of adenylate cycle V and VI (ACV/VI), β_1-adrenergic (β_1-AR) and β_2-adrenergic (β_2-AR) receptor expression (n=6 per group). Western blots were scanned from film with a laser densitometer (Molecular Dynamics), and quantified using ImageQuant software (*Molecular Dynamics*). Values are mean±SEM. *P< 0.05.

DETAILED DESCRIPTION OF THE INVENTION

Modulation of PI3Kgamma has utility in treating a wide range of heart disease, such as heart attack and heart failure. Blocking PI3Kgamma protein activity or gene

expression increases heart function, for example, by providing increased heart muscle contractility. Accordingly, the present invention includes all uses that relate to the realization of the modulatory properties of PI3Kgamma including, but not limited to, the development of therapeutic and diagnostic assays and compositions as well as the preparation and/or isolation of other molecules that inhibit PI3Kgamma.

I. Therapeutic Methods

(a) *Preventing or Treating Heart Disease with small molecules, oligonucleotides or antibodies*

In one aspect, the present invention provides a method of preventing or treating heart disease comprising administering an effective amount of an inhibitor of PI3Kgamma to an animal in need of such treatment. The invention includes a use of an effective amount of an inhibitor of PI3Kgamma for preventing or treating heart disease or to prepare a medicament for preventing or treating heart disease.

The term "PI3Kgamma" or "PI3Kγ" means phosphoinositide 3-kinase gamma and includes any and all forms of this kinase from any source. The term includes biologically active fragments of PI3γ. In a preferred embodiment, the PI3γ includes the catalytic subunit p110gamma (p110γ).

Administration of an "effective amount" of the inhibitor of PI3Kgamma of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. The effective amount of the inhibitor of PI3Kgamma of the invention may vary according to factors such as the disease state, age, sex, and weight of the animal. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The term "animal" as used herein includes all members of the animal kingdom including mammals such as humans.

The term "treatment or treating" as used herein means an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more

symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treating" can also mean prolonging survival as compared to expected survival if not receiving treatment.

The term "an inhibitor of PI3Kgamma" means any molecule or compound that can inhibit the expression of the PI3Kgamma gene or that can inhibit the activity of the PI3Kgamma protein. The inhibitor can be any type of molecule including, but not limited to, proteins (including antibodies), peptides, peptide mimetics, nucleic acids (including DNA, RNA, antisense oligonucleotides, peptide nucleic acids), carbohydrates, organic compounds, small molecules, natural products and library extracts. The inhibitors may be known or novel compounds, including derivatives of the known compounds.

Known inhibitors include wortmannin, Ly294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) and quercetin (see US patent application no. 20020037276 (Ptasznik et al) and CA 2378650 (Forti)). Derivatives and analogues of these compounds are also useful.

In another embodiment, the PI3Kgamma inhibitor is an antibody that binds PI3Kgamma or an antisense oligonucleotide that inhibits the expression of PI3Kgamma. RNA that is complimentary to a nucleic acid sequence from a PI3Kgamma gene can be used in the methods of the present invention to inhibit PI3Kgamma.

Consequently, the present invention provides a method of preventing or treating heart disease comprising administering an effective amount of an antibody or antisense oligonucleotide to an animal in need thereof. Detailed discussion of oligonucleotides and antibodies follows.

RNA interference

RNA interference techniques, such as antisense RNA or siRNA are useful to block gene expression.

Examples of antisense compounds for inhibition of PI3K expression are described in US patent application no. 20020058638 (Monia et al.). Nucleic acid sequences for RNA inhibition of PI3Kgamma could be made from [SEQ ID NO:1] or fragments thereof.

The term "antisense oligonucleotide" as used herein means a nucleotide sequence that is complimentary to its target.

siRNA, refers to double stranded RNA (dsRNA) which silences expression of genes having high identity to either of the strands in the RNA duplex (Fire et al. (1998) Nature 391, 806-811). dsRNA directs gene-specific, post-transcriptional silencing in many mammals through mRNA degradation. siRNA may include nucleic acids modified in the same manner as the oligonucleotide nucleic acids described below.

The term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages. The term also includes modified or substituted oligomers comprising non-naturally occurring monomers or portions thereof, which function similarly. Such modified or substituted oligonucleotides may be preferred over naturally occurring forms because of properties such as enhanced cellular uptake, or increased stability in the presence of nucleases. The term also includes chimeric oligonucleotides which contain two or more chemically distinct regions. For example, chimeric oligonucleotides may contain at least one region of modified nucleotides that confer beneficial properties (e.g. increased nuclease resistance, increased uptake into cells), or two or more oligonucleotides of the invention may be joined to form a chimeric oligonucleotide.

The oligonucleotides of the present invention may be ribonucleic or deoxyribonucleic acids and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The oligonucleotides may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl

guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

Other oligonucleotides of the invention may contain modified phosphorous, oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. For example, the antisense oligonucleotides may contain phosphorothioates, phosphotriesters, methyl phosphonates, and phosphorodithioates. In an embodiment of the invention there are phosphorothioate bonds links between the four to six 3'-terminus bases. In another embodiment phosphorothioate bonds link all the nucleotides.

The oligonucleotides of the invention may also comprise nucleotide analogs that may be better suited as therapeutic or experimental reagents. An example of an oligonucleotide analogue is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyamide backbone which is similar to that found in peptides (P.E. Nielsen, et al Science 1991, 254, 1497). PNA analogues have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. PNAs also bind stronger to a complimentary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other oligonucleotides may contain nucleotides containing polymer backbones, cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Pat. No. 5,034,506). Oligonucleotides may also contain groups such as reporter groups, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an antisense oligonucleotide. Oligonucleotides may also have sugar mimetics.

The nucleic acid molecules may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. The nucleic acid molecules of the invention or a fragment thereof, may be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed with mRNA or the native gene e.g. phosphorothioate derivatives

and acridine substituted nucleotides. The sequences may be produced biologically using an expression vector introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which sequences are produced under the control of a high efficiency regulatory region, the activity of which may be determined by the cell type into which the vector is introduced.

Antibodies

In another embodiment, the inhibitor of PI3Kgamma is a PI3Kgamma specific antibody. Antibodies to PI3Kgamma may be prepared using techniques known in the art such as those described by Kohler and Milstein, Nature 256, 495 (1975) and in U.S. Patent Nos. RE 32,011; 4,902,614; 4,543,439; and 4,411,993, which are incorporated herein by reference. (See also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference). Within the context of the present invention, antibodies are understood to include monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, and $F(ab')_2$) and recombinantly produced binding partners.

The therapeutic methods of the invention can be used to treat any condition wherein it is desirable to modulate PI3Kgamma expression or activity. Such conditions include heart disease caused by acute coronary syndromes (eg. congestive heart failure, angina, myocardial infarction (heart attack)), cardiac arrythmias (eg. atrial flutter, atrial fibrillation, paroxysmal supraventricular tachycardia) and hypertension (eg. idiopathic hypertrophic subaortic stenosis).

In one embodiment, the present invention provides a method of preventing or treating heart disease comprising administering an effective amount of an inhibitor of PI3Kgamma to the recipient animal. The invention includes a use of an effective amount of an inhibitor of PI3Kgamma to prevent or treat heart disease or to prepare a medicament to prevent or treat heart disease.

The above described methods for preventing or treating heart disease using PI3Kgamma inhibitors may be further enhanced by co-administering other drugs, such as beta-blockers.

(b) ***Preventing or treating heart disease with competitively inhibiting protein fragments***

In another aspect, the present invention provides a method of preventing or treating heart disease comprising administering an effective amount of a competitively inhibiting PI3Kgamma protein fragment or a nucleic acid sequence encoding a PI3Kgamma protein fragment to an animal in need thereof.

The term "competitively inhibiting PI3Kgamma protein" includes an inactive full length PI3Kgamma protein as well as inactive fragments or portions of the protein, such as p110gamma, that competitively inhibit binding of PI3Kgamma to its substrates. Preferred fragments or portions of the protein are those that are sufficient to prevent or treat heart disease. The PI3Kgamma protein or the nucleic acid encoding the PI3Kgamma protein can be readily obtained by one of skill in the art. For example, many PI3Kgamma sequences are available in the GenBank database including the human PI3Kgamma [SEQ ID NO:2]. The PI3Kgamma protein or nucleic acid may be modified from the known sequences to make it more useful in the methods of the present invention. Competitive inhibitors, such as inactive peptide fragments, may be designed based on the pI3Kgamma, preferably p110gamma, sequence. One may also use polypeptides having sequence identity to a fragment of pI3Kgamma or p110. The invention also includes polypeptides which have sequence identity at least about: >20%, >25%, >28%, >30%, >35%, >40%, >50%, >60%, >70%, >80% or >90% more preferably at least about >95%, >99%, to a polypeptides fragment of the invention, such as a fragment of [SEQ ID NO:2]. Identity is calculated according to methods known in the art. Sequence identity is most preferably assessed by the BLAST version 2.1 program advanced search (parameters as above; Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403_410). BLAST is a series of programs that are available online at http://www.ncbi.nlm.nih.gov/BLAST. The advanced blast search (http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=1) is set to

default parameters. (ie Matrix BLOSUM62; Gap existence cost 11; Per residue gap cost 1; Lambda ratio 0.85 default).

In one embodiment, the PI3Kgamma protein fragment is prepared as a soluble fusion protein. The fusion protein may contain a domain of PI3Kgamma linked to an immunoglobulin (Ig) Fc Region. The PI3Kgamma fusion may be prepared using techniques known in the art. Generally, a DNA sequence encoding the extracellular domain of PI3Kgamma is linked to a DNA sequence encoding the Fc of the Ig and expressed in an appropriate expression system where the PI3Kgamma - FcIg fusion protein is produced. The PI3Kgamma protein may be obtained from known sources or prepared using recombinant DNA techniques. The protein may have any of the known published sequences for PI3Kgamma. For example, the sequences can be obtained from GenBank as described above. The protein may also be modified to contain amino acid substitutions, insertions and/or deletions. Conserved amino acid substitutions involve replacing one or more amino acids of the PI3Kgamma amino acid sequence with amino acids of similar charge, size, and/or hydrophobicity characteristics. Non-conserved substitutions involve replacing one or more amino acids of the PI3Kgamma amino acid sequence with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

The PI3Kgamma protein may be modified. For example, disulphide bonds may be formed between two appropriately spaced components having free sulfhydryl groups. The bonds may be formed between side chains of amino acids, non-amino acid components or a combination of the two. In addition, the PI3Kgamma protein or peptides of the present invention may be converted into pharmaceutical salts by reacting with inorganic acids including hydrochloric acid, sulphuric acid, hydrobromic acid, phosphoric acid, etc., or organic acids including formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, succinic acid, malic acid, tartaric acid, citric acid, benzoic acid, salicylic acid, benzenesulphonic acid, and tolunesulphonic acids.

The present invention also includes peptide mimetics of the PI3Kgamma of the invention. "Peptide mimetics" are structures which serve as substitutes for peptides in interactions between molecules (See Morgan et al (1989), Ann. Reports Med. Chem.

24:243-252 for a review). Peptide mimetics include synthetic structures which may or may not contain amino acids and/or peptide bonds but retain the structural and functional features of a peptide, or enhancer or inhibitor of the invention. Peptide mimetics also include peptoids, oligopeptoids (Simon et al (1972) Proc. Natl. Acad, Sci USA 89:9367); and peptide libraries containing peptides of a designed length representing all possible sequences of amino acids corresponding to a peptide of the invention.

Peptide mimetics may be designed based on information obtained by systematic replacement of L-amino acids by D-amino acids, replacement of side chains with groups having different electronic properties, and by systematic replacement of peptide bonds with amide bond replacements. Local conformational constraints can also be introduced to determine conformational requirements for activity of a candidate peptide mimetic. The mimetics may include isosteric amide bonds, or D-amino acids to stabilize or promote reverse turn conformations and to help stabilize the molecule. Cyclic amino acid analogues may be used to constrain amino acid residues to particular conformational states. The mimetics can also include mimics of inhibitor peptide secondary structures. These structures can model the 3-dimensional orientation of amino acid residues into the known secondary conformations of proteins. Peptoids may also be used which are oligomers of N-substituted amino acids and can be used as motifs for the generation of chemically diverse libraries of novel molecules.

Peptides of the invention may also be used to identify lead compounds for drug development. The structure of the peptides described herein can be readily determined by a number of methods such as NMR and X-ray crystallography. A comparison of the structures of peptides similar in sequence, but differing in the biological activities they elicit in target molecules can provide information about the structure-activity relationship of the target. Information obtained from the examination of structure-activity relationships can be used to design either modified peptides, or other small molecules or lead compounds that can be tested for predicted properties as related to the target molecule. The activity of the lead compounds can be evaluated using assays similar to those described herein.

Information about structure-activity relationships may also be obtained from co-crystallization studies. In these studies, a peptide with a desired activity is crystallized in association with a target molecule, and the X-ray structure of the complex is determined. The structure can then be compared to the structure of the target molecule in its native state, and information from such a comparison may be used to design compounds expected to possess.

II. Screening Assays

The present invention also includes the isolation and/or identification of substances to inhibit PI3Kgamma expression or activity for treatment of heart disease. Such substances or PI3Kgamma modulators may be useful in the above described therapeutic methods. Two examples of PI3Kgamma modulators include antibodies and antisense molecules which are described in detail above. Other PI3Kgamma modulators may be identified, for example, using the screening assays described below.

(a) Substances that Bind PI3Kgamma and are Useful to Treat Heart Disease

Substances that affect PI3Kgamma activity can be identified based on their ability to bind to PI3Kgamma.

Substances which can bind with the PI3Kgamma of the invention may be identified by reacting the PI3Kgamma with a substance which potentially binds to PI3Kgamma, and assaying for complexes, for free substance, or for non-complexed PI3Kgamma, or for activation of PI3Kgamma. In particular, a yeast two hybrid assay system may be used to identify proteins which interact with PI3Kgamma (Fields, S. and Song, O., 1989, Nature, 340:245-247). Systems of analysis which also may be used include ELISA.

Accordingly, the invention provides a method of identifying substances which can bind with PI3Kgamma, comprising the steps of:

(a) reacting PI3Kgamma and a test substance, under conditions which allow for formation of a complex between the PI3Kgamma and the test substance,

(b) assaying for complexes of PI3Kgamma and the test substance, for free substance or for non complexed PI3Kgamma, wherein the presence of complexes indicates that the test substance is capable of binding PI3Kgamma; and

(c) determining whether the test substance is capable of increasing heart function, such as heart muscle contractility, and thereby prevent or treat heart disease.

Conditions which permit the formation of substance and PI3Kgamma complexes may be selected having regard to factors such as the nature and amounts of the substance and the protein.

The substance-protein complex, free substance or non-complexed proteins may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, antibody against PI3Kgamma or the substance, or labeled PI3Kgamma, or a labeled substance may be utilized. The antibodies, proteins, or substances may be labeled with a detectable substance as described above.

The invention also makes it possible to screen for antagonists that inhibit the effects of an agonist of PI3Kgamma. Thus, the invention may be used to assay for a substance that competes for the same binding site of PI3Kgamma.

(b) Drug Screening Methods

In accordance with one embodiment, the invention enables a method for screening candidate compounds for their ability to decrease the activity of a PI3Kgamma protein. The method comprises providing an assay system for assaying PI3Kgamma activity, assaying the activity in the presence or absence of the candidate or candidate compound and determining whether the compound has decreased PI3Kgamma activity. The method further comprises determining whether the compound is suitable for preventing or treating heart disease.

Accordingly, the present invention provides a method for identifying a compound that inhibits PI3Kgamma protein activity or expression comprising:

(a) incubating a candidate compound with a PI3Kgamma protein or a nucleic acid encoding a PI3Kgamma protein;

(b) determining an amount of PI3Kgamma protein activity or expression and comparing with a control (i.e. in the absence of the test substance), wherein a change in the PI3Kgamma protein activity or expression as compared to the control indicates that the candidate compound has an effect on PI3Kgamma protein activity or expression; and

(c) determining whether the test substance is capable of increasing heart function, such as heart muscle contractility, and thereby prevent or treat heart disease.

In accordance with a further embodiment, the invention enables a method for screening candidate compounds for their ability to decrease expression of a PI3Kgamma protein. The method comprises putting a cell with a candidate compound, wherein the cell includes a regulatory region of a PI3Kgamma gene operably joined to a reporter gene coding region, and detecting a change in expression of the reporter gene.

In one embodiment, the present invention enables culture systems in which cell lines which express the PI3Kgamma gene, and thus PI3Kgamma protein products, are incubated with candidate compounds to test their effects on PI3Kgamma expression. Such culture systems can be used to identify compounds which downregulate PI3Kgamma expression or its function, through the interaction with other proteins.

Such compounds can be selected from protein compounds, chemicals and various drugs that are added to the culture medium. After a period of incubation in the presence of a selected candidate compound(s), the expression of PI3Kgamma can be examined by quantifying the levels of PI3Kgamma mRNA using standard Northern blotting procedure to determine any changes in expression as a result of the candidate compound. Cell lines transfected with constructs expressing PI3Kgamma can also be used to test the function of compounds developed to modify the protein expression. In addition, transformed cell lines expressing a normal PI3Kgamma protein could be mutagenized by the use of mutagenizing agents to produce an altered phenotype in

which the role of mutated PI3Kgamma can be studied in order to study structure/function relationships of the protein products and their physiological effects.

Accordingly, the present invention provides a method for identifying a compound that affects the binding of a PI3Kgamma protein and a PI3Kgamma binding protein comprising:

(a) incubating (i) a candidate compound; (ii) a PI3Kgamma protein and (iii) a PI3Kgamma binding protein under conditions which permit the binding of PI3Kgamma protein to the PI3Kgamma binding protein; and

(b) assaying for complexes of PI3Kgamma protein and the PI3Kgamma binding protein and comparing to a control (i.e. in the absence of the test substance), wherein a reduction of complexes indicates that the compound has an effect on the binding of the PI3Kgamma protein to a PI3Kgamma binding protein;

(c) determining whether the test substance is capable of increasing heart function, such as heart muscle contractility, and thereby prevent or treat heart disease.

All testing for novel drug development is well suited to defined cell culture systems which can be manipulated to express PI3Kgamma and study the result of PI3Kgamma protein modulation. Animal models are also important for testing novel drugs and thus may also be used to identify any potentially useful compound affecting PI3Kgamma expression and activity and thus physiological function.

III. Compositions

The invention also includes pharmaceutical compositions containing substances that inhibit PI3Kgamma inhibitors for use in heart disease as well. Substances that inhibit PI3Kgamma include substances that inhibit PI3Kgamma gene expression as well as substances that inhibit PI3Kgamma activity. Such substances include antisense molecules to PI3Kgamma, antibodies to PI3Kgamma as well as other substances or PI3Kgamma antagonists identified using the screening assays described herein.

Such pharmaceutical compositions can be for intralesional, intravenous, topical, rectal, parenteral, local, inhalant or subcutaneous, intradermal, intramuscular, intrathecal, transperitoneal, oral, and intracerebral use. The composition can be in liquid, solid or semisolid form, for example pills, tablets, creams, gelatin capsules, capsules, suppositories, soft gelatin capsules, gels, membranes, tubelets, solutions or suspensions.

The pharmaceutical compositions of the invention can be intended for administration to humans or animals. Dosages to be administered depend on individual needs, on the desired effect and on the chosen route of administration.

The pharmaceutical compositions can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985).

On this basis, the pharmaceutical compositions include, albeit not exclusively, the active compound or substance in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids. The pharmaceutical compositions may additionally contain other agents to prevent or treat heart disease.

In one embodiment, the pharmaceutical composition for use in preventing or treating heart disease comprises an effective amount of an inhibitor of PI3Kgamma in admixture with a pharmaceutically acceptable diluent or carrier. The PI3Kgamma inhibitor is optionally an antisense oligonucleotide to PI3Kgamma or an antibody that binds to PI3Kgamma.

Pharmaceutical compositions comprising antisense nucleic acid molecules may be directly introduced into cells or tissues *in vivo* using delivery vehicles such as retroviral vectors, adenoviral vectors and DNA virus vectors. They may also be introduced into cells *in vivo* using physical techniques such as microinjection and electroporation or chemical methods such as coprecipitation and incorporation of

DNA into liposomes. Recombinant molecules may also be delivered in the form of an aerosol or by lavage. The nucleic acid molecules of the invention may also be applied extracellularly such as by direct injection into cells.

In another aspect, the pharmaceutical composition for use in preventing or treating heart disease comprises an effective amount of a PI3Kgamma protein or a nucleic acid encoding a PI3Kgamma protein in admixture with a pharmaceutically acceptable diluent or carrier.

IV. Diagnostic Assays

The finding that PI3Kgamma is involved in heart disease allows the detection of conditions involving an increase in PI3Kgamma activity or expression resulting in heart disease.

Accordingly, the present invention provides a method of detecting a condition associated with increased PI3Kgamma expression or activity comprising assaying a sample for (a) a nucleic acid molecule encoding a PI3Kgamma protein or a fragment thereof or (b) a PI3Kgamma protein or a fragment thereof and comparing the amount of PI3Kgamma nucleic acid or protein detected with a suitable control.

(a) Nucleic acid molecules

Nucleotide probes can be prepared based on the sequence of PI3Kgamma for use in the detection of nucleotide sequences encoding PI3Kgamma or fragments thereof in samples, preferably biological samples such as cells, tissues and bodily fluids. The probes can be useful in detecting the presence of a condition associated with PI3Kgamma or monitoring the progress of such a condition. Accordingly, the present invention provides a method for detecting a nucleic acid molecules encoding PI3Kgamma comprising contacting the sample with a nucleotide probe capable of hybridizing with the nucleic acid molecule to form a hybridization product, under conditions which permit the formation of the hybridization product, and assaying for the hybridization product.

A nucleotide probe may be labelled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as 32P,

3H, 14C or the like. Other detectable substances which may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and chemiluminescence. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleic acid to be detected and the amount of nucleic acid available for hybridization. Labelled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual (2nd ed.). The nucleotide probes may be used to detect genes, preferably in human cells, that hybridize to the nucleic acid molecule of the present invention preferably, nucleic acid molecules which hybridize to the nucleic acid molecule encoding PI3Kgamma under stringent hybridization conditions as described herein.

Nucleic acid molecules encoding a PI3Kgamma protein can be selectively amplified in a sample using the polymerase chain reaction (PCR) methods and cDNA or genomic DNA. It is possible to design synthetic oligonucleotide primers from the nucleotide sequence of PI3Kgamma for use in PCR. A nucleic acid can be amplified from cDNA or genomic DNA using oligonucleotide primers and standard PCR amplification techniques. The amplified nucleic acid can be cloned into an appropriate vector and characterized by DNA sequence analysis. cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., Biochemistry, 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

(b) Proteins

The PI3Kgamma protein may be detected in a sample using antibodies that bind to the protein as described in detail above. Accordingly, the present invention provides a method for detecting a PI3Kgamma protein comprising contacting the sample with an antibody that binds to PI3Kgamma which is capable of being detected after it becomes bound to the PI3Kgamma in the sample.

Antibodies specifically reactive with PI3Kgamma, or derivatives thereof, such as enzyme conjugates or labeled derivatives, may be used to detect PI3Kgamma in various biological materials, for example they may be used in any known immunoassays which rely on the binding interaction between an antigenic determinant of PI3Kgamma, and the antibodies. Examples of such assays are radioimmunoassays, enzyme immunoassays (e.g. ELISA), immunofluorescence, immunoprecipitation, latex agglutination, hemagglutination and histochemical tests. Thus, the antibodies may be used to detect and quantify PI3Kgamma in a sample in order to determine its role in particular cellular events or pathological states, and to diagnose and treat such pathological states.

In particular, the antibodies of the invention may be used in immuno-histochemical analyses, for example, at the cellular and sub-subcellular level, to detect PI3Kgamma, to localise it to particular cells and tissues and to specific subcellular locations, and to quantitate the level of expression.

Cytochemical techniques known in the art for localizing antigens using light and electron microscopy may be used to detect PI3Kgamma. Generally, an antibody of the invention may be labelled with a detectable substance and PI3Kgamma may be localised in tissue based upon the presence of the detectable substance. Examples of detectable substances include various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include radioactive iodine I-125, I-131 or 3-H. Antibodies may also be coupled to electron dense substances, such as ferritin or colloidal gold, which are readily visualised by electron microscopy.

Indirect methods may also be employed in which the primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity for the antibody reactive against PI3Kgamma. By way of example, if the antibody

having specificity against PI3Kgamma is a rabbit IgG antibody, the second antibody may be goat anti-rabbit gamma-globulin labelled with a detectable substance as described herein.

Where a radioactive label is used as a detectable substance, PI3Kgamma may be localized by autoradiography. The results of autoradiography may be quantitated by determining the density of particles in the autoradiographs by various optical methods, or by counting the grains.

Other Modulation of PI3Kgamma and PTEN

One may also activate PI3Kgamma or block PTEN for excessive heart contractility. The invention includes a method of treating heart disease, such as excessive heart contractility, in a mammal in need thereof by administering an agent that activates PI3Kgamma or blocks PTEN. RNA compounds, antibodies and other molecules may be used to activate PI3Kgamma or block PTEN. Such compounds may be prepared as described in this application. For example, PTEN may be blocked by using nucleic acids based on [SEQ ID NO:3] or competitive inhibitors based on [SEQ ID NO:4].

The invention also includes the use of an inhibitor of PTEN or an activator of PI3Kgamma to prepare a medicament to prevent or treat heart disease, such as excessive contractility. The inhibitor of PTEN may comprise inactive PTEN, an inactive fragment of PTEN, an antibody to PTEN or a nucleic acid that inhibits the expression of PTEN, for example by RNA interference. The activator of PI3K could include an antibody or other molecule. The invention also includes a pharmaceutical composition for use in preventing or treating heart disease, such as excessive contractility, comprising an inhibitor of PTEN or activator of PIK3gamma and a carrier.

The invention also includes a method for identifying a compound that activates the binding of a PI3Kgamma protein to its substrate for treatment of heart disease, such as increased contractility, comprising:

(a) incubating (i) a candidate compound; (ii) an PI3Kgamma protein and (iii) an PI3Kgamma substrate under conditions which permit the binding of PI3Kgamma protein to the substrate; and

(b) assaying for complexes of PI3Kgamma protein and the substrate or metabolites thereby produced and comparing to a control, wherein an increase of complexes or metabolites indicates that the candidate compound has an effect on the binding of the PI3Kgamma protein to the substrate;

(c) determining whether the candidate compound is useful for treatment of heart disease.

The invention also includes a compound identified by this method.

The invention also includes a method for identifying a compound that inhibits the binding of a PTEN protein to its substrate for treatment of heart disease, such as increased contractility, comprising:

(d) incubating (i) a candidate compound; (ii) an PTEN protein and (iii) an PTEN substrate under conditions which permit the binding of PTEN protein to the substrate; and

(e) assaying for complexes of PTEN protein and the substrate or metabolites thereby produced and comparing to a control, wherein a reduction of complexes or metabolites indicates that the candidate compound has an effect on the binding of the PTEN protein to the substrate;

(f) determining whether the candidate compound is useful for treatment of heart disease, such as increased contractility.

The invention also includes a compound identified by this method.

Inhibition of PI3K and/or activation of PTEN blocks cardiac hypertrophy. The invention also includes the use of an inhibitor of PI3K or an activator of PTEN to prepare a medicament to prevent or treat heart disease, such as excessive contractility or hypertrophy. The inhibitor of PI3K may comprise inactive PI3K, an inactive fragment of PI3K, an antibody to PI3K or a nucleic acid that inhibits the expression of PI3K, for example by RNA interference. The activator of PTEN could include an

antibody or other molecule. The invention also includes a pharmaceutical composition for use in preventing or treating heart disease, such as excessive contractility, comprising an inhibitor of PI3K or activator of PTEN and a carrier.

The invention also includes a method for identifying a compound that activates the binding of a PTEN protein to its substrate for treatment of heart disease, such as hypertrophy, comprising:

(g) incubating (i) a candidate compound; (ii) a PTEN protein and (iii) a PTEN substrate under conditions which permit the binding of PTEN protein to the substrate; and

(h) assaying for complexes of PTEN protein and the substrate or metabolites thereby produced and comparing to a control, wherein an increase of complexes or metabolites indicates that the candidate compound has an effect on the binding of the PTEN protein to the substrate;

(i) determining whether the candidate compound is useful for treatment of heart disease, such as hypertrophy.

The invention also includes a compound identified by this method.

The invention also includes a method for identifying a compound that inhibits the binding of a PI3K protein to its substrate for treatment of heart disease, such as or hypertrophy, comprising:

(j) incubating (i) a candidate compound; (ii) a PI3K protein and (iii) a PI3K substrate under conditions which permit the binding of PI3K protein to the substrate; and

(k) assaying for complexes of PI3K protein and the substrate or metabolites thereby produced and comparing to a control, wherein a reduction of complexes or metabolites indicates that the candidate compound has an effect on the binding of the PI3K protein to the substrate;

(l) determining whether the candidate compound is useful for treatment of heart disease, such hypertrophy.

The invention also includes a compound identified by this method.

One may also modulate hypertrophy in a mammal in need thereof by administering an agent that modulates PTEN or PI3Kgamma. The invention includes a method of treating heart disease, such as hypertrophy, in a mammal in need thereof, by administering an agent that activates PTEN or inhibits PI3Kgamma.

Peptide fragments, antibodies, RNA and other compounds described in this application are useful for the above methods of medical treatment. The above diagnostic methods and other methods of the invention described in the application may also be adapted for use with the methods described in this section.

EXAMPLES

Example 1

Experimental Results

Generation of cardiac muscle specific PTEN knockout mice

Inactivation of PTEN in all cells results in early embryonic lethality between day 6.5 to 9.5 (Suzuki et al, 1998; Di Cristofano et al, 1998). We therefore used a tissue specific gene targeting approach to mutate PTEN in all cardiomyocytes and skeletal muscle cells. Mice that have exon 4 and 5 of PTEN flanked by loxP sites by homologous recombination ($PTEN^{flox}$) (Suzuki et al, 2001), were mated with a CRE deleter line that expresses the CRE transgene under the control of the muscle creatinine kinase promoter (mckCRE) (Bruning et al, 1998). We generated homozygous mutant mice that express CRE and are floxed at both PTEN alleles (mckCRE-$PTEN^{\Delta/\Delta}$), heterozygous mice that express CRE but are only floxed at one PTEN allele (mckCRE-$PTEN^{\Delta/+}$), and control mice that do not express mckCRE but have both PTEN alleles floxed ($PTEN^{flox/flox}$). No phenotypic differences between mckCRE-$PTEN^{\Delta/+}$ and $PTEN^{flox/flox}$ genotypes were observed. Western blotting showed a significant reduction in the amount of PTEN protein in both the heart as well as skeletal muscle, but no other tissues, of mckCRE-$PTEN^{\Delta/\Delta}$ mice (Figure 1A). The presence of residual PTEN protein in mckCRE-$PTEN^{\Delta/\Delta}$ mice is due to PTEN expression in cardiac non-myocyte cell types such as endothelial cells or fibroblasts.

Hearts from mckCRE-PTEN$^{\Delta/\Delta}$ mice showed no alteration in the expression of PI3Kα, PI3Kβ, PI3Kγ catalytic subunits nor changes in p85α expression (Figure 1B).

Spontaneous cardiac hypertrophy in PTEN-deficient hearts

Analysis of skeletal muscle from mckCRE-PTEN$^{\Delta/\Delta}$ mice showed no gross abnormalities or any overt changes in cell size compared to control mckCRE-PTEN$^{\Delta/+}$ or PTEN$^{flox/flox}$ littermates. Overall body weights were comparable between all three genotypes at all ages analyzed (10 wks, 6 months and 12 months). Moreover, there was no indication of diabetes and blood glucose levels did not differ between mckCRE-PTEN$^{\Delta/\Delta}$, mckCRE-PTEN$^{\Delta/+}$, and PTEN$^{flox/flox}$ littermates.

Whereas skeletal muscle appeared normal, loss of PTEN in cardiac muscle resulted in greatly increased heart sizes in the mutant mckCRE-PTEN$^{\Delta/\Delta}$mice (Figure 1C, D). Concordantly, there was a significant increase in heart/body weight ratios in the PTEN-deficient hearts indicative of cardiac hypertrophy at 10 weeks and 12 months of age (Figure 2A). The increased heart size was already detectable in newborn mckCRE-PTEN$^{\Delta/\Delta}$mice. Histologically, the increase in heart size was observed throughout the heart (Figure 1D). Interestingly, cardiac hypertrophy did not result in fibrotic or structural changes in myocyte organization (Figure 1D) or perturbations in cardiomyocyte cell death even at 1 year of age showing that loss of PTEN does not result in cardiac decompensation or dilated cardiomyopathy.

To further show the cardiac hypertrophy in PTEN-deficient hearts, cardiomyocytes were isolated from adult mice to assess the individual cell size. Loss of PTEN resulted in a marked increase in both the length and width of cardiomyocytes as compared to controls (Figure 2B). The length-to-width ratio was unchanged in these cells showing that the cell size change was similar to that observed in physiologic hypertrophy (Hunter and Chien, 1999). Pathological cardiac hypertrophy is characterized by a prototypical change in gene expression patterns such as increased ANF, βMyHC, BNP, and skeletal-actin expression, and a decrease in αMyHC (Hunter and Chien, 1999). However, no significant alterations in the expression profile of these hypertrophic genes were observed with the exception of an increase in βMyHC

(Figure 2C, D). Thus, loss of PTEN in the myocardium induces hypertrophy without the typical pathological changes in the gene expression profiles or cardiac decompensation.

Activation of PI3K signaling in PTEN-deficient hearts

Since PTEN antagonizes the action of PI3K, we showed that the loss of PTEN in the hearts results in the activation of downstream targets of PI3K signaling (Downward, 1998). Loss of PTEN resulted in a significant increase in the basal phosphorylation state of AKT/PKB (Figure 2E, F). In addition, we found an increase in the phosphorylation of GSK3β and $p70^{S6K}$, both downstream targets of PI3K signaling (Figure 2E, F). No apparent changes in the basal activation of ERK1/ERK2 were observed between the mutant mckCRE-$PTEN^{\Delta/\Delta}$ mice and their mckCRE-$PTEN^{\Delta/+}$ and $PTEN^{+/+}$ littermates (Figure 2E, F). Loss of PTEN in cardiomyocytes results in increased PI3K signaling leading to activation and phosphorylation of multiple PI3K target molecules.

Loss of PTEN in the heart results in decreased cardiac contractility

To further show characterize the role of PTEN in mutant mckCRE-$PTEN^{\Delta/\Delta}$ mice, we analyzed the hearts using echocardiography. Consistent with the physiological cardiac hypertrophy, echocardiography of PTEN-deficient hearts revealed an increase in the anterior wall thickness and an increase of the left ventricle mass (LVM) at all ages analyzed (Figure 3A and Table 1). Moreover, the change in end diastolic dimension of the left ventricle (LVEDD) reflected this overall enlargement of PTEN-deficient heart. No difference in heart rate was detected in the PTEN-deficient hearts (Table 1).

Surprisingly, assessment of cardiac function by echocardiography revealed that mutant mckCRE-$PTEN^{\Delta/\Delta}$ mice exhibited a dramatic reduction in cardiac contractility characterized by decreased fractional shortening (FS), decreased velocity of circumferential fibre shortening (Vcfc) and reduced peak aortic outflow velocity (PAV) (Figure 3B,C and Table 1). To confirm the echocardiographic alterations, functional invasive hemodynamic measurements were performed. Our hemodynamic measurements showed that both dP/dT-max and dP/dT-min were markedly reduced in

the PTEN-mutant mice (Table 1), again indicating severe impairment of contractile heart function. Despite marked reductions in contractility at 10 weeks, there was no further decrease in the function of the PTEN-deficient hearts analyzed at 12 months of age (Table 1). In addition, there was no evidence of dilation, wall thinning or tissue fibrosis in older animals (Figure 1D, Table 1). PTEN controls cardiomyocyte size and heart muscle contractility.

Increased contractility in PI3Kγ mutant mice

The decrease in cardiac contractility found in the PTEN-mutant hearts suggested that increased PI3K signaling serves not only to increase cell size in the heart but to also suppress basal heart function. Neither the p110α dominant negative nor p110α constitutively active heart specific transgenic animals showed any alteration in heart function (Shioi et al, 2000) suggesting that the effect on contractility is mediated by PI3K isoforms other than PI3Kα. Several GPCR signaling pathways, including adrenergic receptors (Rockman et al, 2002), are important mediators of cardiac function. Decreased heart function in PTEN mutant mice can be mediated by PI3Kγ. We observed p110γ protein expression in total heart extracts of mice (Figure 1B) and in isolated cardiomyocytes (Figure 3D). Loss of p110γ expression does not alter the expression levels of p85, p110α, and p110β (Figure 3E). Moreover, p85 associated PI3K activity in cardiomyocytes was comparable between $p110\gamma^{-/-}$ and wild type littermates using in vitro lipid kinase assays (not shown). There was also no apparent difference in the expression or phosphorylation of AKT/PKB (Figure 3E). Mice deficient for p110γ were found to have no alteration in heart size or left ventricle mass (LVM) and displayed normal heart rates (Figure 3A, F and Table 1). No structural alterations were observed in the hearts of $p110\gamma^{-/-}$ mice using histological analyses and there were no changes in the gene expression profiles of the cardiac hypertrophy markers ANF, βMyHC, BNP, skeletal-actin, and αMyHC. Thus, whereas loss of PTEN results in cardiac hypertrophy, P13Kγ has no role in the control of basal cardiomyocyte size.

The hearts of p110γ deficient mice displayed a marked enhancement in contractility as assessed by increased fractional shortening (FS), Vcfc, and peak aortic outflow velocity (PAV) (Figure 3B,C and Table 1). Hemodynamic measurements confirmed

the increased heart contractility in p110$\gamma^{-/-}$ mice (Table 1). Telemetry analysis of blood pressure in conscious mice revealed normal blood pressure in p110$\gamma^{-/-}$ mice. In addition, plasma epinephrine and norepinephrin levels were not altered in p110$\gamma^{-/-}$ mice compared to controls (norepinephrin (59.63 ± 9.99 in wild type (n=6) versus 61.28 ± 8.82 in p110$\gamma^{-/-}$ mice (n=6), and epinephrin (4.65 ± 0.80 in wild type (n=6) versus 4.28 ± 0.55 in p110$\gamma^{-/-}$ mice (n=6)).

To show the long term effects of this increase in cardiac function in p110γ deficient mice, we analyzed heart function by echocardiography in 1 year old mice. Similar to that observed in younger mice, there remained an increase in cardiac contractility (fractional shortening (48.49 ± 0.76 in wild type (n=6) vs. 55.16 ± 0.33 in p110$\gamma^{-/-}$ mice (n=6), and Vcfc (8.65 ± 0.20 in wild type (n=6) versus 10.86 ± 0.17 in p110$\gamma^{-/-}$ mice (n=6)). To ensure that the increase in cardiac contractility observed in the p110γ was due to the disruption of the gene and not an aberrant gene targeting event, we analyzed heart function in a second independently generated mouse line deficient for p110γ (Hirsch et al, 2000). All alterations in heart functions analyzed were similar between the two independent p110γ deficient mouse lines. Specifically, the second p110γ mouse line (Hirsch et al, 2000) had increased contractility as assessed by fractional shortening (48.07 ± 0.68 in wild type (n=6) vs. 56.47 ± 0.87 in p110$\gamma^{-/-}$ mice (n=6), and Vcfc (8.62 ± 0.16 in wild type (n=6) versus 11.35 ± 0.28 in p110$\gamma^{-/-}$ mice (n=6)). Moreover, hypercontractility persisted (Vcfc: 8.65 ± 0.2 in wild type versus 10.86 ± 0.17 in p110$\gamma^{-/-}$ mice; n= 6; $p<0.01$) and there was no evidence of cardiac hypertrophy or increased myocardial fibrosis in 1 year old mice. Thus, loss of PI3Kγ results in a marked increase in cardiac contractility.

PI3Kγ mediates reduction of heart function in PTEN-deficient hearts

The alterations in heart muscle contractility in p110γ– and PTEN-mutant hearts were exactly opposite, that is loss of PTEN resulted in decreased heart functions whereas loss of p110γ leads to a marked enhancement in cardiac contractility. To show that the role of PTEN in regulating cell size and heart function could be genetically uncoupled, we generated mice deficient for both p110γ and PTEN in the heart. Hearts

from mckCRE-PTEN$^{\Delta/\Delta}$ p110$\gamma^{-/-}$ double mutant mice were significantly enlarged as compared to wild type and p110$\gamma^{-/-}$ mutant mice (Figure 3A, Table 1). The extent of cardiac hypertrophy in mckCRE-PTEN$^{\Delta/\Delta}$ p110$\gamma^{-/-}$ double mutant mice was similar to that observed for hearts from mckCRE-PTEN$^{\Delta/\Delta}$single mutants (Figure 3A). Histologically, hearts from mckCRE-PTEN$^{\Delta/\Delta}$ p110$\gamma^{-/-}$ double mutant mice were also similar to that of mckCRE-PTEN$^{\Delta/\Delta}$single mutant mice. Importantly, similar to PTEN-deficient hearts, AKT/PKB was hyperphosphorylated in the hearts of mckCRE-PTEN$^{\Delta/\Delta}$ p110$\gamma^{-/-}$ double mutant mice (Figure 3G). Thus, loss of PI3Kγ in PTEN-deficient hearts has no affect on the increased AKT/PKB phosphorylation and does not rescue the cardiac hypertrophy phenotype.

However, unlike PTEN-deficient hearts, mckCRE-PTEN$^{\Delta/\Delta}$ p110$\gamma^{-/-}$ double mutant hearts were found to be hypercontractile, as assessed by increased fractional shortening (FS), Vcfc, and peak aortic outflow velocity (PAV) (Figure 3B,C, Table 1). These improved heart functions were similar to that of p110γ single mutant hearts. The defect in cardiac contractility seen in the PTEN-deficient hearts is dependent on the PI3Kγ signaling pathway. Importantly, PTEN regulated hypertrophy can be genetically uncoupled from the cardiac contractility changes mediated by PTEN-PI3Kγ.

Reversal of cardiac hypertrophy in PTEN-deficient hearts by a dominant-negative p110α transgene

The increase in cell size found in PTEN-deficient hearts strongly resembles that seen in transgenic mice for heart specific constitutively active p110α that couples to receptor tyrosine kinases (Shioi et al, 2000). Furthermore, mice transgenic for heart-specific dominant negative p110α (DN-p110a) have smaller than normal heart size. We therefore generated mice deficient for PTEN in the hearts and overexpressing the heart specific DN-p110α transgene. Hearts of mckCRE-PTEN$^{\Delta/\Delta}$; DN-p110α mice displayed a reduction in heart size similar to that seen in single DN-p110α transgenic mice (Fig. 4A,B). The presence of the DN-p110α transgene also correlated with a decrease in the basal level of AKT/PKB activation in the absence or presence of a

functional PTEN gene (Figure 4C). However, whereas overexpression of DN-p110α on a wild type background affects heart size but has no apparent effect on heart function, hearts from mckCRE-PTEN$^{\Delta/\Delta}$; DN-p110α displayed a marked decrease in contractility (Figure 4D, E and Table 1). PI3Kα signaling modulates basal cell size of cardiomyocytes while PI3Kγ signaling controls basal cardiac contractility.

PI3Kγ and PTEN control contractility in single cells

To further analyse these *in vivo* contractility defects, we showed that loss of PTEN and PI3Kγ can affect contractility of a single adult cardiomyocyte *in vitro*. In agreement with both the *in vivo* echocardiographic and hemodynamic data, it was found that PTEN deficient cardiomyocytes display a reduction in contractility as determined by a reduction in percent cell shortening and positive and negative dL/dTs as compared to wild type cardiomycoytes (Figure 5A,B; Table 2). Conversely, p110γ deficient myocytes displayed an increase in contractility (Figure 5A,B; Table 2). Cardiomyocytes deficient for both PTEN and p110γ display increased contractility despite the hypertrophy present in these cells (Figure 5A,B; Table 2). Moreover, treatment of wild type myocytes with the PI3K inhibitor LY294002 lead to a significant increase in cardiomyocyte contractility. In p110γ deficient cardiomyocytes addition of LY294002 had no effect on the already increased contractility (Figure 5B; Table 2). These *in vitro* data show that independent of exogenous agonists PTEN and PI3Kγ control basal cardiomyocyte contractility within a single cell.

Other inhibitors described in this application also trigger the same result as that obtained in the cells (KO cells) with wortmannin and LY294002.

PI3Kγ modulates baseline cAMP levels in cardiomyocytes

One intriguing aspect of the *in vitro* contractility data is that there was a significant increase in the rate of relaxation in p110γ deficient cardiomyocytes compared to control cells despite a marked increase in peak contraction (Table 2). Cardiac relaxation is modulated via cAMP dependent pathways, where cAMP activates PKA which then inhibits phospholamban (PLB) leading to increased sarcaplasmic reticulum calcium ATPase (SERCA) activity (Brittsan and Kranias, 2000). Moreover,

it has previously been shown in vascular smooth muscle cells that pharmacological inhibition of PI3K can lead to increases in cAMP levels (Komalavilas et al, 2001). To show how alterations in cAMP levels can be contributing to the phenotype observed here, we measured baseline cAMP levels in isolated myocytes. In unstimulated PTEN-deficient cardiomyocytes, cAMP levels were significantly reduced (Figure 5C). Conversely, an increase in cAMP levels was detected in isolated cardiomyocytes deficient for p110γ (Figure 5C). Treatment of PTEN-deficient and wild-type cardiomyocytes with the PI3K inhibitor Wortmannin resulted in a marked increase of cAMP to levels observed in p110γ deficient cells (Figure 5C). Induction of cAMP production in response to the adenylate cyclase agonist forskolin was comparable among all genotypes analyzed (Figure 5D) showing that the basal machinery for cAMP production is operational in all genotypes. PI3Kγ and PTEN modulate cAMP production in cardiomyocytes.

To show how the alterations in cAMP levels contribute to altered contractility, we measured contractility in single cells following blockade of cAMP-dependent activation of downstream targets using Rp-cAMP. In wild type adult mouse cardiomyocytes, treatment with Rp-cAMP lead to a marked decrease in contractility (Figure 5E; Table 2). Importantly, treatment of p110γ deficient cardiomyocytes with the cAMP blocker lead to a greater reduction in contractility as compared to wild type cells such that the difference in the resultant contractility of wild type and p110γ deficient cells treated with Rp-cAMP was greatly reduced compared to untreated cells (Figure 5E; Table 2). In addition, the rate of relaxation in p110γ deficient cardiomyocytes treated with Rp-cAMP was reduced to wildtype levels (Table 2). Alterations in baseline cAMP levels contribute to the increased contractility in p110γ$^{-/-}$ hearts.

PI3Kγ and β-adrenergic signaling

It has been previously shown that PI3Kγ can bind to GPCR-kinase 2 (GRK2) (Naga Prasad et al, 2001a), a kinase that mediates desensitization of GPCRs like β-adrenergic receptors (β-AR) (Lefkowitz, 1998). Thus, β-adrenergic signaling may be affected in p110γ$^{-/-}$ hearts, and alterations of PI3K activity may affect GRK activity

and/or β-AR expression. Using an *in vitro* kinase assay, GRK activity in both cytosolic and membrane-associated fractions of PTEN-deficient hearts was comparable to that of control hearts (Figure 6A). Total protein levels of GRK2 were also comparable (Figure 6A). Radiolabeled ligand binding assays showed that there is also no detectable difference in the density of β-AR receptors or their affinity for the β-AR ligand ICYP in the PTEN- or p110γ-deficient hearts as compared to wild-type mice (Figure 6B). These data imply that changes in contractility of PTEN and p110γ-deficient hearts are not due to alterations in baseline β-adrenergic receptor levels or the modulation of GRK activity.

The G-protein coupled β_2-adrenergic receptors (β_2-AR) are linked to both Gαs and Gαi G-proteins that can either increase (Gαs) or decrease (Gαi) cAMP levels and heart muscle contractility (Kuznetsov et al, 1995; Xiao et al, 1999; Rockman et al, 2002). To determine if PI3Kγ can modulate cAMP production following GPCR stimulation, we purified neonatal cardiomyocytes from both wild type and p110$\gamma^{-/-}$ hearts and stimulated these cells with the β_2-AR selective agonist zinterol. As described previosuly for adult rat myocytes (Kuznetsov et al, 1995), stimulation of the β_2-AR had no affect on cAMP levels in wild type mouse cardiomyoctes. By contrast, selective stimulation of the β_2-AR in p110$\gamma^{-/-}$ cardiomyocytes resulted in a marked increase in cAMP levels (Figure 6C). Conversely, stimulation of β1-AR receptors resulted in a similar increase in cAMP levels in both wild type and p110γ-deficient cardiomyocytes demonstrating selectivity for the action of PI3Kγ on cAMP production (Figure 6D).

In cardiomyocytes, increased cAMP levels result in enhanced contractility via activation of PKA and subsequent phosphorylation of phospholamban (PLB) in the sarcoplasmic reticulum (Brittsan and Kranias, 2000). Similar to cAMP production, no alteration of PLB phosphorylation could be detected in wild type cells upon stimulation with the β2-AR agonist zinterol (Figure 6E). However, when *p110*$\gamma^{-/-}$ cardiomyocytes were stimulated with the same β2-AR agonist, a dose dependent increase in PLB phosphorylation was observed. Thus, in addition to its role in baseline cardiac contractility, loss of p110γ expression releases the inhibition of the

β2-AR thereby permitting receptor-induced cAMP production and subsequent PLB-phosphorylation.

The invention shows the roles of PI3K-PTEN signaling in cardiac hypertrophy and heart functions in PTEN-heart muscle specific mutant mice, p110γ knock-out, dominant-negative p110α transgenic mice, and double-mutant mice. The tyrosine kinase-receptor p110α-PTEN pathway is a critical regulator of cardiac cell size. The PTEN-PI3Kγ signaling pathway regulates heart muscle contractility via GPCRs. This contractility phenotype is present in single cardiomyocytes and dependent on cAMP signaling. PI3Kγ regulates cardiac function by negatively regulating cAMP levels and phospholamban phosphorylation upon β_2-adrenergic receptor stimulation. These data show that the tumor suppressor PTEN has an important *in vivo* role in GPCR signaling and that PTEN and PI3Kγ signaling control heart function.

PTEN and heart muscle size

The cardiac hypertrophy in PTEN deficient hearts is similar to that in mice overexpressing a constitutively active p110α transgene in the heart (Shioi et al, 2000). Like these transgenic mice, the hypertrophy found in PTEN deficient hearts displayed features characteristic of physiological hypertrophy, such as increase in both the length and width of the myocytes, no fibrotic changes and no decompensation into dilated cardiomyopathy (Hunter and Chien, 1999). The reversal of heart size in the PTEN deficient hearts that expressed a dominant negative PI3Kα transgene indicates that it is this isoform of PI3K that modulates basal cell size in cardiomyocytes. Moreover, it does not appear that any other PI3K isoform can compensate for the loss of PI3Kα function *in vivo*. It should be pointed out that it is possible that this transgene interferes with not only p110α, but also p110β and p110δ as all of these require p85 activity which is likely sequestered as a result of the transgene expression. Nevertheless, there is a differential action of class Ia and class Ib PI3Ks as it is unlikely that this transgene interferes with p110γ function. This is further supported by the absence of any observable alteration in cardiac contractility in mice expressing the DN-p110α transgene.

Previously, PI3K signaling has been implicated in cell size regulation in other tissues and organisms such as *drosophila* (Scanga et al, 2000). Mice deficient for $p70^{S6K}$ have reduced body size due to a reduction in cell size (Shima et al, 1998). Recently, brain specific disruptions of PTEN have been reported which result in the increase in neuronal cell size (Backman et al, 2001). Moreover, transgenic expression of activated AKT/PKB in mouse heart leads to a hypertrophic phenotype and this phenotype can be inhibited with a DN-p110α transgene (Shioi et al, 2002; Matsui et al, 2002). This is consistent with our data on the differential modulation of AKT/PKB phosphorylation in PTEN but not PI3Kγ mutant hearts. Thus, it appears that AKT/PKB and $p70^{S6K}$ signaling plays an important role in the regulation of basal cardiomyocyte size *in vivo*.

Our results show that PI3Kγ has no role in the homeostatic control of cardiomyocyte cell size. Yet many agonists used to induce PI3K-dependent cardiomyocyte hypertrophy *in vitro* are GPCR agonists (Rabkin et al, 1997; Schluter et al, 1999). Moreover, PI3Kγ is activated in pressure overload induced hypertrophy *in vivo* (Naga Prasad et al, 2000), implying a role for PI3Kγ in agonist-induced cardiac hypertrophy. We determine the role of PI3Kγ in agonist-induced hypertrophy *in vivo* following stimulation of GPCRs.

Regulation of heart muscle contractility by PI3Kγ and PTEN

PTEN deficient hearts showed cardiac hypertrophy and surprisingly also displayed a dramatic decrease in cardiac contractility. Conversely, PI3Kγ deficient hearts exhibited an increase in cardiac contractility. PI3Kγ-PTEN signaling controls heart muscle function in vivo. PI3Kγ can modulate contractility in the absence of exogenous agonists. These contractility changes are at least in part mediated by alteration in cAMP levels.

Most likely the phenotypes observed here are due to the lipid kinase/phosphatase activity of these enzymes. Hearts deficient in both PTEN and PI3Kγ also displayed increased cardiac contractility showing that increased PIP3 generated via PI3Kγ causes the decrease in cardiac contractility seen in the PTEN deficient hearts.

Moreover, the different receptors recruit different downstream targets for the same phospholipids.

β2 adrenergic receptors are coupled to both Gαs and Gαi signaling (Rockman et al., 2002). The opposing effects of these two G protein subunits result in no global net effect on cardiac contractility or cAMP production upon receptor stimulation (Kuznetsov et al, 1995; Xiao et al, 1999). It has been shown, however, that if the Gαi subunit is inhibited, a Gαs response and an increase in myocyte contractility ensues (Xiao et al, 1999). Our results indicate that loss of PI3Kγ in heart muscle cells relieves an inhibition resulting in cAMP production and PLB phosphorylation upon β2 AR agonist stimulation consistent with an inhibition of Gαi. In this scenario, loss of PI3Kγ would result in a lack of Gαi cAMP inhibition and a subsequent increase in cAMP levels and PLB phosphorylation resulting in the inhibition of PLB and increased SERCA function effectively enhancing contractility (Zvaritch et al, 2000; Frank and Kranias, 2000). Additional molecular mechanisms controlling cAMP levels and PLB phosphorylation in a PTEN-PI3Kγ dependent manner cannot be excluded.

In heart failure, β1-AR densities decrease leading to a greater prominence of β2 adrenergic signaling (Naga Prasad et al, 2001b). Furthermore, it has been shown that increased PLB activity is a critical regulator of decreased contractility in dilated cardiomyopathy (Minamisawa et al, 1999). It is likely that if PI3Kγ is upregulated in heart failure, and, it is known that p110γ expression can be induced in pressure overload hypertrophy (Naga Prasad et al, 2000). In the presence of increased PI3Kg, b2 adrenergic signaling leads to a reduction in cardiac contractility. Inhibition of PI3Kg will lead to better cardiac contractility in heart failure.

To further study remodeling in the hearts histological analysis was carried out. In control hearts treatment with isoproterenol lead to disorganization of the myocytes and a significant increase in cardiac fibrosis. Quantification of interstitial fibrosis showed a significant increase in collagen deposition in isoproterenol treated control hearts. This increased fibrosis and disorganization is consistent with the decompensation of the hypertrophic myocardium and the progression to dilated

cardiomyopathy. In contrast to control hearts, p110gamma deficient hearts diplayed no increase in cardiac fibrosis and no alteration in myocyte organization.

Cardiac hypertrophy is associated with a prototypical alteration in gene expression profiles. In models of cardiomyocyte hypertrophy in culture, PI3K has been shown to play a role in the onset of these alterations in gene expression. To determine if loss of p110gamma alters the changes in gene expression seen in isoproterenol induced cardiac hypertrophy, we assed the mRNA levels of hypertrophy markers. Isoproterenol has been shown to induce significant alterations in gene expression albeit blunted in comparison to other models of hypertrophy. Consistently, we observed a prototypical increase in ANF and BNP expression in control hearts. We also observed a slight decrease in a-myosin heavy chain expression. In p110gamma deficient hearts a similar alteration in expression profiles was found compared to control hearts, despite the attenuation of hypertrophy. This would suggest that p110gamma may not be required for alteration in expression profile seen in cardiac hypertrophy, and that alterations in gene expression profiles can be uncoupled from cardiac hypertrophy.

The uncoupling of expression profiles from cardiac hypertrophy further suggests that these alterations in gene expression profile may not be sufficient to induce cardiac hypertrophy. Previously it has been shown that increase in endogenous activation of PKB/AKT is associated with increases in cardiomyocyte size, and overexpression of a constitutively active form of PKB/AKT could induce an increase in heart size. PKB/AKT is a major downstream effector of PI3K signaling, suggesting that AKT activation may play a role in the cardiac hypertrophy observed here. To determine if AKT activation was associated with the isoproterenol induced cardiac hypertrophy, we assessed AKT activation using an in vitro kinase assay. In control hearts, chronic stimulation with isoproterenol led to a significant increase in AKT kinase activity. However, in p110gamma deficient hearts, chronic isoproterenol infusion did not increase endogenous levels of AKT activity. This data correlates well with the observed attenuation of cardiac hypertrophy seen in PI3Kgamma deficient hearts and suggests that AKT activation may play a critical role in the hypertrophic response to isoproterenol infusion.

Inhibition of PI3K gamma improves function in a model of heart failure

A role for PI3K in cardiomyocyte hypertrophy has been demonstrated upon agonist stimulation in cultured cells. Previously we have shown that PI3Kalpha modulates basal cell size in cardiomyocytes while loss of PI3Kgamma results in increased cardiac contractility with no effect on basal cell size. However, in many instances where PI3K signaling has been implicated in cardiac hypertrophy, GPCR agonists have been used for the induction of hypertrophy suggesting that PI3Kgamma the isoform linked to GPCR signaling, may play a role in agonist induced cardiac hypertrophy. Moreover, the increased cardiac contractility seen in PI3Kgamma deficient hearts suggest a potential role for PI3Kgamma inhibition in the treatment of heart failure. To show the role for PI3Kgamma in agonist induced hypertrophy and cardiomyopathy, we induced cardiac hypertrophy and subsequent impairment of pump function by chronic infusion of isoproterenol, a beta-adrenergic agonist in PI3Kgamma deficient mice. Here we show that PI3Kgamma deficient mice have an attenuated hypertrophic response to isoproterenol induced cardiac hypertrophy *in vivo* and that PI3Kgamma deficient mice retain their increase in pump function following this chronic beta-adrenergic receptor stimulation. These data highlight the in *vivo* role of PI3Kgamma in hypertrophy, and show that PI3Kgamma is a novel therapeutic target for the treatment of decreased pump function in heart failure.

Control hearts treated with isoproterenol displayed an increase in cardiac hypertrophy and a dilation of the left ventricle consistent with the onset of dilated cardiomyopathy. In contrast the architecture of p110gamma deficient hearts remained largely unchanged. Previously we have shown that these p110gamma deficient hearts display an increase in cardiac hypertrophy. It has been suggested that this increase in pump function may serve to improve cardiac performance in models of dilated cardiomyopathy. We assessed cardiac function in control and PI3Kgamma deficient hearts with and without the induction of hypertrophy using echocardiography. In control hearts the chronic infusion of isoproterenol lead to a significant reduction in systolic heart function. Both fractional shortening and velocity of circumferential fiber shortening were significantly reduced in these hearts. This data is consistent with the increased interstitial fibrosis and dilation of the left ventricle observed in

these hearts, and strongly supports the onset on dilated cardiomyopathy in these hearts. On the other hand, chronic isoproterenol stimulation of p110gamma deficient hearts did not significantly reduce cardiac function and these hearts retained an increase pump function relative to untreated control hearts.

We have shown here that PI3Kgamma plays a role in isoprorternol induced cardiac hypertrophy. It has been previously shown that PI3Kg is activated in pressure overload hypertrophy but that this increase in activity was not required for the hypertrophic response. This suggests that PI3Kgamma may not be required for all forms of cardiac hypertrophy but possibly may play a critical role in hypertrophy induced through GPCR mediated pathways. Our data here suggests that this hypertrophy may be in part mediated by activation of PKB/AKT pathways. Recently it has been shown that overexpression of an activated form of AKT could induce cardiac hypertrophy in vivo. It has also been shown that PI3Kgamma is essential for ERK activation in some systems. Transgenic mouse models with heart specific increases in ERK activation have been shown to lead to a physiologic hypertrophy. Thus a MAP kinase mediated pathway may also play a role in the hypertrophy observed here. Nevertheless we were unable to detect alterations in phospho-MAPK in isoproterenol induced hypertrophic hearts. Thus the role of MAPK activation in our model of hypertrophy remains unclear.

In control hearts isoproternol infusion led to a decrease in cardiac function and the onset of cardiomyopathy. In p110gamma deficient hearts, however, displayed a resistance to any overt effects on pump function induced by isoproterenol. This data represents the first evidence for a potential therapeutic role of modulation of PI3K signaling in heart failure. We have shown that the increase in cardiac contractility in PI3Kgamma deficient hearts is mediated by an increase in phospholamban inhibition. It has been shown that modulation of PLB activity either directly or indirectly can rescue numerous models of heart failure. Currently there is a great interest in determining the validity of PLB modulation as a therapeutic target for heart failure indications as a way to safely improve pump function, an aspect of heart failure not sufficiently attenuated by current therapeutics. While the assessment of the effect PI3Kgamma inhibition has in other genetic and acquired models of heart failure is

required it is interesting to speculate that specific inhibitors of PI3Kgamma may prove to be useful therapeutics for heart failure indications. Importantly, however, it will be necessary to alter PLB activity following the onset of heart failure, as opposed to prior to phenotypic onset to properly assess its potential as a therapeutic modality.

The invention shows that inhibition of PI3Kgamma improves function in a model of heart failure. Table 3 below shows this result. Mice were treated with isoproterenol chronically for 7 days. In control +/- animals heart function is reduced, but in -/- heart function is maintained.

Experimental Procedures

Mutant mice. The generation and genotyping of the PTEN-floxed, mckCRE, p110γ, and DN-p110α mice have been previously described (Suzuki et al, 2001; Bruning et al, 1998; Sasaki et al, 2000; Shioi et al, 2000; Hirsch et al, 2000). Only littermate mice were used as controls. Mice were bred and maintained following institutional guidelines.

Protein and mRNA expression analyses. Northern and Western blotting were carried out as described (Crackower et al, 2002) using antibodies to PTEN (Cascade), p110α, p110β, p110γ (Santa Cruz), p85 (Upstate Biotechnologies), phospho-AKT/PKB (S473), AKT/PKB, phospho-GSK3β, GSK3β, phospho-$p70^{S6K}$, phospho-ERK1/2, ERK1/2, phospho-phospholamban (Serine16), phospholamban (Cell Signaling Technology), and GAPDH (Research Diagnostics). For immunoprecipitations, lysates were incubated with anti-GRK2/3 Abs (Upstate Biotechnologies) conjugated with Sepharose beads for 16 hours at 4°C. Immunoprecipitates were separated using SDS–PAGE gel electrophoresis, transferred onto membranes and immunoblotted.

Heart morphometry, echocardiography, and hemodynamic measurements. For heart morphometry, hearts were arrested with KCL, fixed with 10% buffered formalin and embedded in paraffin. Fibrosis was analyzed using trichrome staining and quantitative morphometry using color-subtractive computer assisted image analysis. Echocardiographic assessments and invasive hemodynamic measurements were

carried out as described (Crackower et al, 2002). Catecholamines were extracted from the plasma of male mice using alumina, eluted and then assayed using HPLC (Waters Associates Inc., Milford, MA, USA) coupled with electrochemical detection (ESA Inc., Bedford, MA, USA). The intra-assay and inter-assay coefficient of variation were <5% and <10%, respectively.

Cardiomyocyte purification. Adult ventricular myocyte were isolated as described (Sah *et al.*, 2002). Cell size was measured using Openlab 2.2.5 software (Improvision Ltd). Ventricular myocytes were placed in a Plexiglas chamber and continuously perfused with oxygenated Tyrodes buffer (137mM NaCl, 5.4mM KCl, 10mM glucose, 10mM HEPES, 0.5mM NaH_2PO_4, 1mM $MgCl_2$, 1.2mM $CaCl_2$, and pH 7.4) at 2.5ml/min at 36°C. Neonatal ventricular cardiac myocytes were isolated from 1-3 day old mice in CBFHH buffer (137mM NaCl, 5.36mM KCl, 0.81mM $MgSO_4 \cdot 7H_2O$, 5.55mM dextrose, 0.44mM KH_2PO_4, 0.34mM $Na_2HPO_4 \cdot 7H_2O$, 20μM Hepes, pH 7.4) as described (Steinberg et al, 1991). Experiments were carried out 48-72 hrs after plating.

Contractility assays and cAMP measurements. Cardiomyocytes were stimulated at 1 Hz with a Grass S44 stimulator (pulse duration 3 ms; 15-20 volts) and a video edge detector (Crescent Electronics) was used to track myocyte contractions at 240Hz. Steady state contractions were recorded at 1kHz following a 4 mins equilibrium period using a Phillips 800 camera system (240Hz) and Felix acquisition software (Photon Technologies Inc.). Cardiomyocyte length, percent fractional shortening, shortening rate (+dL/dT), and relaxation rate (−dL/dT) were determined at baseline. Myocytes were incubation with LY294002 (30μM) (Calbiochem) and Rp-cAMPS (25μM) (Sigma/RBI) for 10 minutes and recordings were made during continuous perfusion of the drug dissolved in Tyrode. In all experiments phosphodiesterase activity was inhibited by pre-treating the cells with theophylline (100mM) for 30 min prior to stimulation. Wortmannin treatment (100nM) was initiated at the time of theophylline addition. Zinterol (Bristol-Myers Squibb) stimulation was carried out at RT for 10 min. Forskolin (Sigma) was used at 25mM. Isoproterenol (Sigma) stimulation was carried out at RT for 10 min, following 5 min. pretreatment of ICI

118,551 (Sigma/RBI) (10^{-7}M). cAMP was measured by RIA according to the supplier's protocol (Amersham).

GRK activity and b-Adrenergic Receptor binding. GRK activity was measured by rhodopsin phosphorylation as described (Louden et al., 2000). Hearts were homogenized in 20mM Tris pH 7.3, 0.8mM $MgCl_2$, 2mM EDTA. Membrane fractions were isolated by centrifuging at 40,000*g* for 30min at 4°C. 300µg and 20µg of protein were assayed for cytosolic and membrane fractions, respectively. b-AR density and affinity for its ligand ICYP (3-Iodocyanopindolol [^{125}I]) were determined by equilibrium radio-ligand binding assays on sarcolemmal membranes prepared from ventricular myocardium as described (Steinberg et al., 1995). Briefly, assays were performed with 30mg membrane protein and ICYP (5-250pM) in the absence or presence of 0.1mM unlabeled propranolol to determine non-specific binding in a final volume of 1ml for 60min at 37°C. ICYP bound to membranes was separated from free, unbound ICYP by rapid vacuum filtration over glass-fiber filters (Gelman A/E).

Histology Methods

Mice. P110gamma deficient mice have previously been described. Hypertrophy was induced by chronic infusion of isoproterenol (sigma) for 7 days at a dose of 15mg/Kg/day using alzet osmotic minipumps. Mice were aneastitized with O2/isoflourane and the pumps were inserted subcutaneously at the level of the scapula. The incision was closed and mice were allowed to recover. Pumps were removed 24 hrs. prior to echocardiographic analysis.

Heart morphometry, echocardiography, hemodynamics and blood pressure measurements For heart morphometry, hearts were arrested with KCL and fixed with 10% buffered formalin. and subsequently embedded in paraffin. Myocardial interstitial fibrosis was determined using trichrome stained sections and quantitative morphometry using color-subtractive computer assisted image analysis (Image Processing Tool Kit version 2.5). Echocardiographic assessments were performed as described (Wickenden et al, 1999). Briefly, mice were anesthetized with isoflourane/oxygen and examined by transthoracic echocardiography using a Acuson® Sequoia C256 equipped with a 15MHz linear transducer. (FS) was

calculated as: FS = [(EDD – ESD)/EDD] x 100. Vcfc was calculated as FS/ejection time corrected for heart rate.

Protein Kinase and mRNA expression analyses

Total RNA was prepared form hearts using trizol ( ). 20 mg of RNA was resolved on a 0.8% formamide gel, blotted to nylon membranes (Amersham), and probed with cDNA probes for ANF, BNP, αMHC, and GAPDH. AKT kinase activity was measured from whole hearts according to manufacturer's protocols (Upstate).

Example 2

PI3Kγ is critical for the induction of hypertrophy, fibrosis, and cardiac dysfunction function in response to β-adrenergic receptor stimulation in vivo. Thus, PI3Kγ is a novel therapeutic target for the treatment of decreased cardiac function in heart failure. We infused isoproterenol, a β-adrenergic receptor agonist, into PI3Kγ deficient mice. Compared to controls, isoproterenol infusion in PI3Kγ deficient mice resulted in an attenuated cardiac hypertrophic response and markedly reduced interstitial fibrosis. Intriguingly, chronic β-adrenergic receptor stimulation triggered impaired heart functions in wild type mice, whereas PI3Kγ deficient mice retained their increased heart function and did not develop heart failure. The lack of PI3Kγ attenuated the activation of Akt/PKB and ERK1/2 signaling pathways in cardiac myocytes in response to isoproterenol. Beta1- and beta2-adrenergic receptor densities were decreased by similar amounts in PI3Kγ deficient and control mice showing that PI3Kγ isoform plays no role in the downregulation of beta-adrenergic receptors following chronic beta-adrenergic stimulation.

Methods

Mice. *p110γ*$^{-/-}$ mice have previously been described (Sasaki ete al., 2000). Hypertrophy and heart failure were induced by chronic infusion of isoproterenol (Sigma) for 7 days at a dose of 15mg/kg/day using Alzet® osmotic mini-pumps. Pumps were removed 24h prior to echocardiography and hemodynamic measurements. All experiments were performed in accordance to institutional guidelines. Only littermate male mice of 10-12 weeks of age were used.

Heart morphometry, TUNEL assay, echocardiography, and hemodynamic measurements. For heart morphometry, hearts were arrested with KCl, fixed with 10% buffered formalin, and embedded in paraffin. Myocardial interstitial fibrosis was determined as collagen volume fraction (PSR-stained sections) using color-subtractive computer assisted image analysis (Image Processing Tool Kit version 2.5). In situ DNA fragmentation was labeled using the TUNEL assay (ApopTag Plus kit) (Oncor, Gaithersburg, MD). Echocardiographic assessments and invasive hemodynamic measurements were carried out as described.(Crackower et al. 2002).

Signaling, mRNA and Western Blot analyses. Total RNA was prepared form hearts using Trizol, resolved on a 0.8% formamide gel, blotted to nylon membranes (Amersham), and probed with cDNA probes for ANF, BNP, aMHC, and GAPDH. Akt/PKB kinase activity was measured using a commercial in vitro kinase assay kit according to the manufactures protocol (Upstate Biotechnologies) (Sasaki et al. 2000). Ventricular cardiomyocytes were isolated as described (Crackower et al. 2002). Experiments were carried out 48-72 hrs after plating and removal of fibroblasts. ERK1/2 phosphorylation, p38 phosphorylation, and ERK1/2 protein levels were determined by Western blotting (Cell Signaling Technology).

Beta-adrenergic receptor densities. Mice were anaesthetized with isoflurane/O_2, hearts were removed and ventricular tissue excised and frozen. Samples were processed and light sarcolemmae were not treated (-) or treated with PNG-F (+) and subjected to SDS-PAGE as previously described (Rybin et al. 2000).

Results

To show the role of PI3Kγ in GPCR-induced cardiac hypertrophy and cardiomyopathy, we chronically infused littermate *p110γ*$^{-/-}$ (knockout) and *p110γ*$^{+/-}$ (control) mice with the β-adrenergic receptor agonist, isoproterenol. Control mice displayed a marked increase in heart size (Fig. 9A), heart/body weight and heart/tibial length ratios (Fig. 9B) and left ventricular mass and wall thickness (Table 4). In isoproterenol-infused *p110γ*$^{-/-}$ mice, the hypertrophic response still occurred but was markedly attenuated (Fig. 9A and B) (Table 4). Cardiac hypertrophy is associated with prototypical alterations in gene expression (Hunter et al., 1999). We therefore

assessed the mRNA levels of hypertrophy markers in hearts of $p110g^{+/-}$ and $p110\gamma^{-/-}$ mice chronically stimulated with isoproterenol. Intriguingly, despite the attenuation of hypertrophy in $p110\gamma^{-/-}$ hearts, we observed a similar alteration in expression profiles in ANF, BNP and α-myosin heavy chain compared to $p110\gamma^{+/-}$ hearts (Fig. 9C).

It was unclear whether PI3Kγ is essential for GPCR-signaling in cardiomyocytes (Rockman et al. 2002; Naga Prasad et al. 2002). We therefore stimulated purified ventricular cardiomyocytes from $p110\gamma^{+/-}$ and $p110\gamma^{-/-}$ littermates with the GPCR-agonist, isoproterenol, or the tyrosine kinase-based receptor agonist, basic fibroblast growth factor (bFGF) (Fig. 9D). Whereas bFGF-induced ERK1/2 activation occurred normally, isoproterenol-induced ERK1/2 phosphorylation was completely abolished in $p110\gamma^{-/-}$ cardiomyocytes. Similarly, in $p110\gamma^{-/-}$ hearts, chronic isoproterenol infusion did not increase endogenous Akt/protein kinase B (PKB) activity (Fig. 9E). By contrast, activation of the stress kinase p38 appeared to be normal in both $p110\gamma^{+/-}$ and $p110\gamma^{-/-}$ cardiomyocytes (Fig. 9D).

Given the marked differences in signaling and hypertrophic responses, we showed the functional changes in heart function in mice infused with isoproterenol following withdrawal of the drug. Chronic infusion of isoproterenol into $p110\gamma^{+/-}$ mice lead to a significant reduction in heart functions as assessed by markedly decreased fractional shortening, velocity of circumferential fiber shortening and peak aortic velocity (Table 4). Invasive hemodynamic measurements showed increased left ventricular end-diastolic pressure, and decreases in $\pm dP/dt_{max}$ and blood pressure in $p110\gamma^{+/-}$ animals (Table 4), again indicating severe impairment of heart function. By contrast, chronic isoproterenol stimulation of $p110\gamma^{-/-}$ mice caused a markedly smaller reduction in cardiac function and blood pressures. Moreover, these hearts retained an increased pump function relative to untreated control hearts (Table 4).

Decompensation of the hypertrophic myocardium and the progression to cardiomyopathy is accompanied by increased fibrosis and disorganization of myocytes. We therefore carried out histological analysis to further study the cardiac remodeling response. In control hearts treatment with isoproterenol resulted in disorganization of the myocytes and a marked increase in cardiac fibrosis (Fig. 10A).

Quantification of interstitial fibrosis showed a significant increase in collagen deposition in the hearts of isoproterenol-treated control mice (Fig. 10B). By contrast, *p110γ*$^{-/-}$ mice displayed no significant increase in cardiac fibrosis or alteration in myocyte organization (Fig. 10A,B). Cardiomyocyte cell death was comparable between isoproterenol-treated *p110γ*$^{-/-}$ and control mice (Fig. 10C). Importantly, measurements of cardiomyocyte sizes in cross-sections confirmed impaired cardiomyocyte hypertrophy in *p110γ*$^{-/-}$ mice (Fig. 10D). There were no differential changes in β1- or β2-adrenergic receptor expression in membrane fractions from *p110γ*$^{+/-}$ and *p110γ*$^{-/-}$ hearts at baseline and in response to chronic in vivo isoproterenol stimulation (Fig. 11A and B). Expression levels of the major cardiac adenylate cyclase isoforms V and VI, (ACV/VI), were comparable between *p110γ*$^{+/-}$ and *p110γ*$^{-/-}$ hearts (Fig. 11A and B).

Excessive catecholamines and adrenergic stimulation has been clearly linked to cardiac hypertrophy and disease. Indeed, altered signaling via the G-protein coupled β-adrenergic receptors have been implicated in heart failure in humans (Rockman et al., 2002; Hajjar and MacRae, 2002; Scheuer, 1999; Brede et al., 2002). Loss of *p110γ* leads to an increase in cardiomyocyte contractility. Our data show that PI3Kγ, the PI3K-isoform linked to GPCRs, is critical for the induction of myocardial hypertrophy, interstitial fibrosis, and cardiac dysfunction in response to β-adrenergic receptor stimulation in vivo. Importantly, *p110γ*$^{-/-}$ mice display a resistance to the effects of isoproterenol on cardiac structure and function. An increase in cardiac function may serve to improve myocardial performance in models of dilated cardiomyopathy and we confirmed that *p110γ*$^{-/-}$ hearts maintained their hypercontractility following chronic adrenergic stimulation. However, the induction of several hypertrophy markers in response to isoproterenol was not affected by the loss of *p110γ* which shows that *p110γ* is not required for alteration in fetal gene expression seen in myocardial hypertrophy and that these genetic changes can be uncoupled from the hypertrophic process.

Our data show that chronic activation of β-adrenergic signaling results in markedly decreased heart function and the onset of cardiomyopathy in *p110γ*$^{+/-}$ mice, loss of *p110γ* renders animals resistant to increased interstitial fibrosis and cardiac

dysfunction. Akt/ protein kinase B (PKB) is a major molecular target of the PI3K signaling pathway involved in cell hypertrophy in multiple systems, and the mitogen-activated protein kinase, ERK1/2 also plays a central role in cardiac hypertrophy. Genetic evidence in hematopoietic cells show that PI3Kγ is the sole PI3K that couples to GPCRs. Importantly, infusion of isoproterenol into $p110\gamma^{+/-}$ mice showed that β-adrenergic receptor stimulation induced *in vivo* activation of Akt/PKB and *in vitro* activation ERK1/2. These genetic data demonstrate for the first time that following GPCR stimulation, PI3Kγ is the essential PI3K isoform required for Akt/PKB and ERK1/2 activation in cardiomyocytes. In other models of heart disease (pressure-overload hypertrophy), there is also activation of Akt/PKB via the PI3Kγ isoform. Despite the lack of activation of Akt/PKB and ERK1/2 pathways in $p110\gamma^{-/-}$ mice, which are clearly antiapoptotic pathways, apoptosis was not increased in the $p110\gamma^{-/-}$ mice which is likely related to the antiapoptotic effects mediated by enhanced β_2-adrenergic receptor signaling in $p110\gamma^{-/-}$ cardiomyocytes.

Since PI3K also has a role in GPCR signaling in isolated cardiac fibroblasts (Kim et al., 2002), the reduced interstitial fibrosis in the heart may be the direct consequence of attenuated β-adrenergic receptor-mediated stimulation of cardiac fibroblasts in the $p110\gamma^{-/-}$ hearts. PI3Kg might be involved in β-adrenergic receptor down-regulation (internalization). However, the loss of PI3Kγ has no apparent effect on the down-modulation of β-adrenergic receptors in response to chronic adrenergic stimulation. However, we cannot rule out the role of other PI3K isoforms in the downregualtion of beta-adrenergic receptors and there exists the distinct possibility that receptor desensitization could be differentially affected i.e. functional responses are different. In summary, we have shown that the PI3K5 has an important role to maintain heart function under the pathologic condition of chronic adrenergic stimulation and our results represent the first genetic evidence for a therapeutic role of the modulation of PI3Kγ signaling in heart failure.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not

limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Table 1. Heart functions of mutant mice

	10 weeks		12 months					
	mckCRE -PTEN$^{\Delta/+}$ (wildtype)	*mckCRE -PTEN$^{\Delta/\Delta}$*	*mckCRE -PTEN$^{\Delta/+}$*	*mckCRE -PTEN$^{\Delta/\Delta}$*	*p110γ$^{-/-}$*	*mckCRE-PTEN$^{\Delta/\Delta}$ p110γ$^{-/-}$*	*DN-p110α*	*mckCRE-PTEN$^{\Delta/\Delta}$ DN-p110α*
	n=7	n=7	n=6	n=6	n=7	n=8	n=4	n=4
Heart Rate, bpm	518 ± 13	545 ± 19	545 ± 9	529 ± 18	486 ± 25	535 ± 11	522 ± 7	533 ± 7
AW, mm	0.67 ± 0.03	0.74 ± 0.03 **	0.70 ± 0.03	0.84 ± 0.02 **	0.70 ± 0.02	0.80 ± 0.01**	0.61 ± 0.01*	0.57 ± 0.03*
LVEDD, mm	4.00 ± 0.07	4.35 ± 0.15 *	4.10 ± 0.05	4.32 ± 0.15	3.99 ± 0.11	4.12 ± 0.04*	3.87 ± 0.09	3.85 ± 0.14
LVESD, mm	2.20 ± 0.04	3.11 ± 0.14 **	2.24 ± 0.05	2.92 ± 0.15 *	1.74 ± 0.06**	1.94 ± 0.06*	2.10 ± 0.07	2.57 ± 0.07**
LVM, mg	97.39 ± 2.62	134.04 ± 2.64 **	103.57 ± 4.87	142.93 ± 4.59 **	92.22 ± 6.75	128.87 ± 2.59*	83.18 ± 1.23*	83.55 ± 1.42*
% FS	44.97 ± 0.68	28.39 ± 1.90 **	44.50 ± 1.28	32.86 ± 1.26 **	56.50 ± 0.95**	52.98 ± 1.39**	45.62 ± 0.82	33.26 ± 0.80**
Vcfc, circ/s	8.71 ± 0.10	5.26 ± 0.30 **	9.23 ± 0.18	6.41 ± 0.23 **	11.15 ± 0.34**	10.78 ± 0.36*	9.39 ± 0.22	6.51 ± 0.21**
PAV, m/s	0.940 ± 0.024	0.887 ± 0.023 *	0.826 ± 0.029	0.697 ± 0.019 *	1.017 ± 0.052**	1.116 ± 0.070**	0.888 ± 0.044	0.830 ± 0.024
dP/dT-max	+ 6045 ± 148	+ 4077 ± 243**			+ 9202 ± 473**			
dP/dT-min	- 5274 ± 251	- 3731 ± 158**			- 8036 ± 500**			

*p < 0.05 vs wildtype mice.
**p < 0.01 vs wildtype mice.
Bpm = heart beats per minute; AW = anterior wall thickness; LVEDD = left ventricle end diastolic dimension; LVESD = left ventricle end systolic dimension; LVM = left ventricular mass; %FS = percent fractional shortening; Vcfc = Velocity of circumferential fiber shortening; PAV = peak aortic outflow velocity. dP/dT max = maximum 1st derivative of the change in left ventricular pressure/time; dP/dT min = minimum 1st derivative of the change in left ventricular pressure/time

Table 2. Contractility of isolated single cardiomyocytes

	Wildtype	*mckCRE -PTEN$^{\Delta/\Delta}$*	*p110γ$^{-/-}$*	*mckCRE-PTEN$^{\Delta/\Delta}$ p110γ$^{-/-}$*	*Wildtype (LY294002) (30μM)*	*p110γ$^{-/-}$ (LY294002) (30μM)*	*Wildtype (Rp-CAMPs) (25μM)*	*p110γ$^{-/-}$ (Rp-CAMPs) (25μM)*
RestingLength,μm	121.7 ± 3.1	146.7 ± 2.9 **	123.6 ± 3.2	147.3 ± 2.4 **	123.8 ± 2.1	124.1 ± 3.4	120.6 ± 2.6	122.1 ± 2.2
% Shortening	11.4 ± 0 51	7.1 ± 0.43 **	17.4 ± 1.1 **	16.6 ± 1.2 **	15.6 ± 0.97**	16.5 ± 1.3**	9.68 ± 0.37 **	12.1 ± 0.41†
+dL/dT, μm/s	339 ± 17.2	224 ± 14.3 **	797 ± 45 **	723 ± 42 **	503 ± 22.5**	717 ± 53**	278 ± 12.3 **	498 ± 21.3 †
-dL/dT, μm/s	358 ± 18.1	242 ± 16.4 **	960 ± 56 **	889 ± 58 **	521 ± 25.8**	912 ± 63**	289 ± 17.2 **	579 ± 31.6 †
TPS, ms	55.3 ± 3.2	49.4 ± 3.8	48.7 ± 2.2	50.2 ± 2.4	50.3 ± 2.9	53.3 ± 3.1	54.7 ± 2.8	53.1 ± 2.6
T90R, ms	71.9 ± 3.6	76.7 ± 4.5	54.7 ± 2.8 **	51.3 ± 2.6 **	50.1 ± 2.3**	50.5 ± 2.5**	76.9 ± 3.2	68.8 ± 3.7 †

**p < 0.01 vs wildtype cells.
† p< 0.01 vs untreated p110γ$^{-/-}$
Mean ± SEM are shown; n=15 cells from 3 hearts for each group. +dL/dT max = maximum 1st derivative of the change in cell length/time; -dP/dT = minimum 1st derivative of the change in cell length /time; TPS=time to peak shortening; T90R=time to 90% relaxation.

Table 3. Heart functions of *mckCRE - PTEN$^{\Delta/\Delta}$* mice

	p110γ$^{+/+}$		*p110γ*$^{-/-}$	
	H_2O	Isoproterenol (15mg/Kg/day/7days)	H_2O	Isoproterenol (15mg/Kg/day/7days)
Body Weight, g	29.60 ±1.02	29.45 ±0.59	28.76 ±0.64	29.21 ±0.50
Heart Weight/Body Weight, mg/g	4.62 ±0.11	6.64 ±0.35	4.69 ±0.16	5.39 ±0.15
Heart Rate, bpm	495 ±12	485 ±17	488 ±26	492 ±7
Anterior wall thickness, mm	0.69 ±0.02	0.76 ±0.02	0.70 ±0.02	0.71±0.03
LVEDD, mm	3.98 ±0.12	4.32 ±0.10	3.99 ±0.11	4.14 ±0.10
LVESD, mm	2.22 ±0.09	2.94 ±0.13	1.74 ±0.06	1.93 ±0.09
%FS	44.34 ±1.16	31.22 ±1.49	56.50 ±0.95	53.43 ±1.51
PAV	875 ±0.032	799 ±0.034	1,017 ±0.052	916 ±0.014
VCFC	8.26 ±0.20	6.04±0.41	11.15 ±0.34	10.52 ±0.38
LVMASS	93.25 ±4.91	131.10 ±1.43	92.22 ±6.75	111.65 ±1.41
LVBW	3.16 ±0.18	4.79 ±0.08	3.20 ±0.20	3.85 ±0.06

$^{*}p < 0.05$ mckCRE-PTEN$^{\Delta/\Delta}$ vs mckCRE-PTEN$^{+/+}$ mice.
$^{**}p < 0.01$ mckCRE-PTEN$^{+/+}$ vs mckCRE-PTEN$^{+/+}$ mice.
Bpm = heart beats per minute; AW = anterior wall thickness; LVEDD = left ventricle end diastolic dimension; LVESD = left ventricle end systolic dimension; LVM = calculated left ventricular mass; %FS = percent fractional shortening; Vcfc = Velocity of circumferential fiber shortening; PAV = peak aortic outflow velocity.

Table 4. Echocardiographic and hemodynamic parameters in littermate *p110γ* $^{+/-}$ and *p110γ* $^{-/-}$ mice following chronic β-adrenergic receptor stimulation

	p110γ$^{+/-}$ +Vehicle	*p110γ*$^{-/-}$ Vehicle	*p110γ*$^{+/-}$ +Iso	*p110γ*$^{-/-}$ + Iso
HR (bpm)	521±13.5	503±14.8	491±17.2	513±9.3
AW (mm)	0.693±0.019	0.696±0.015	0.879±0.02^{A}	0.733±0.017
PW (mm)	0.687±0.016	0.706±0.022	0.884±0.021^{A}	0.742±0.018
LVEDD (mm)	3.98±0.11	4.03±0.12	4.15±0.08^{B}	3.96±0.1
LVESD (mm)	2.16±0.09	1.73±0.06	2.89±0.07^{A}	1.95±0.09
LVM/BW (mg/g)	3.16±0.18	3.2±0.17	4.79±0.13^{A}	3.65±0.11^{C}
FS (%)	46.7±1.2	57.8±1.3^{B}	29.6±1.9^{A}	50.3±1.4^{C}
PAV (cm/s)	87.5±3.2	98.9±5.1^{B}	68.6±3.4^{A}	92.5±2.9
VCFc (circ/s)	8.26±0.27	11.3±0.42^{B}	5.43±0.31^{A}	10.3±0.38
SBP (mmHg)	108.3±2.9	112±3.6	75.6±3.3^{A}	107.8±3.2
MABP (mmHg)	83.4±3	85.4±2.2	53.2±2.4^{A}	81.4±2.9
LVEDP (mmHg)	5.84±0.3	5.65±0.44	10.9±0.86^{A}	6.55±0.58
+dP/dt$_{max}$ (mmHg/sec)	+5995±120	+8984±241^{B}	+3142±294^{A}	+7963±257^{C}
-dP/dt$_{min}$ (mmHg/sec)	-5487±128	-8093±257^{B}	-2706±218^{A}	-6987±216^{C}

n=8 for all groups; values are mean±SEM; ^{A}p<0.01 compared with all other groups, ^{B}p<0.01 compared with Control+Vehicle group; ^{C}p <0.05 compared with p110$\gamma^{-/-}$+Vehicle group; HR=Heart Rate; AW, PW=Anterior and Posterior Left Ventricular Wall Thickness; LVEDD, LVESD=Left Ventricular End Diastolic and Systolic Diameter; LVM=Left Ventricular Mass; BW=Body Weight; FS=Fractional Shortening; PAV=Peak Aortic Velocity; VCFc=Velocity of Circumferential Shortening corrected for Heart Rate; SBP=Systolic Blood Pressure; MABP=Mean Arterial Blood Pressure; LVEDP=Left Ventricular End-Diastolic Pressure; +dP/dt$_{max}$ and -dP/dt$_{min}$=maximum and minimum first derivative of the change in left ventricular pressure; Iso = Isoproterenol.

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